

Starch-driven optimisation of manure bioconversion by housefly larvae

L.W. Pisa

Propositions

1. In starch-supplemented chicken excreta, total larval biomass is driven more by survival than by individual larval weight.
   (this thesis)

2. Mean individual larval weight hides more than it reveals.
   (this thesis)

3. Humans will disappear before honeybees.

4. Experimental blocks used as random effects require both a valid population interpretation and statistical support.

5. Scientific and religious authority differ primarily in validation.

6. War protects insect biodiversity from intensive agriculture.

Propositions belonging to the thesis, entitled

Starch-driven optimisation of manure bioconversion by housefly larvae

Lennard Pisa
Wageningen, 25 August 2026

Starch-driven optimisation of manure bioconversion by housefly larvae

L.W. Pisa

Thesis committee

Promotors

Prof. Dr W.H. Hendriks

Professor of Animal Nutrition

Wageningen University & Research

Dr G. Bosch

Associate professor in Animal Nutrition

Wageningen University & Research

Co-Promotor

Dr D.G.A.B. Oonincx

Assistant professor in Animal Nutrition

Wageningen University & Research

Other members

Prof. Dr B. Wertheim, Wageningen University & Research

Dr K. Jensen, Aarhus University, Denmark

Dr C. D. Miranda, Howard Payne University, USA

Dr T. Veldkamp, Wageningen University & Research

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

Starch-driven optimisation of manure bioconversion by housefly larvae

L.W. Pisa

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus,

Prof. Dr C. Kroeze,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Tuesday 25 August 2026

at 3:30 p.m. in the Omnia Auditorium

Lennard Pisa

Starch-driven optimisation of manure bioconversion by housefly larvae

163 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2026)

With references and summary in English

DOI: 10.18174/681012

Table of contents

Chapter 1 General introduction 1
1.1 Opportunities and challenges for housefly larvae mass-rearing 2
1.2 Nutritional physiology, phylogeny and ecology of housefly larvae 8
1.3 Research justification 17
1.4 Thesis aim and theoretical approach 18
1.5 Outline of the thesis 19
Chapter 2 Bioconversion of fresh chicken excreta by housefly larvae (*Musca domestica* L.) in relation to excreta sterilization and carbohydrate addition 31
Chapter 3 Dose-response effects of starch in sterilized and unsterilized chicken excreta on *Musca domestica* larval growth and bioconversion efficiency 49
Chapter 4 Timing of digestible carbohydrate addition to fresh chicken excreta affects housefly (*Musca domestica*) larval growth and bioconversion 67
Chapter 5 Rearing of housefly larvae (*Musca domestica* L.) in different larval densities on chicken excreta supplemented with digestible carbohydrates 89
Chapter 6 General discussion 111
6.1 Introduction 112
6.2 Excreta sterilization, starch addition and larval growth 112
6.3 Timing of starch addition 118
6.4 Starch addition, larval density and larval survival 119
6.5 Bioconversion efficiency across experiments 120
6.6 Limitations and methodological considerations 124
6.7 Implications for chicken excreta nutrient upcycling 129
6.8 Suggestions for future research 129
6.9 Main conclusions 131
Summary 135
Acknowledgements 139
About the author 143
Appendix 1 buoyancy separation of larvae 147
Appendix 2 housefly culture and egg harvest 153

Chapter 1

General introduction

1.1 Opportunities and challenges for housefly larvae mass-rearing

Opportunities for housefly rearing

Animal protein demand for human consumption is high and expected to increase due to population growth, projected to reach 9.7 billion by 2050 (UN DESA 2024). With growing wealth in developing countries, global demand for animal protein is projected to rise 20 to 50% by 2050 compared to 2020 (WEF 2023, Van Eenennaam 2024).

The use of natural resources for livestock production has been stated to reach planetary boundaries, with estimated mammalian livestock biomass (~630 million tons) outweighing wild terrestrial mammalian biomass by a factor 30 (Greenspoon *et al*. 2023). In 2017, 50% of agricultural land (20% of earth's land area), was used for production of livestock feed and grazing (Mottet *et al*. 2017). The demand for fish as a feed ingredient increasingly competes with its use as food and overfishing leads to dwindling fish stocks (Serra *et al*. 2024). Further increases in livestock feed production will increase pressure on agriculture and fisheries that are additionally contributing to pollution and greenhouse gas emissions (Steinfeld *et al*. 2006, FAO 2012, Field *et al*. 2014, Godfray *et al*. 2018). The current scale of livestock production also leads to a local overabundance of livestock manure, creating major challenges in aquatic and terrestrial ecosystems due to excess nitrogen and phosphate (van Beek *et al*. 2004, Godfray *et al*. 2018). Large scale imports of livestock feed to sustain intensive livestock farming in developed nations have decoupled, and continue to decouple, feed and livestock production and strongly contribute to localized manure overabundance (Wang *et al*. 2022).

The Netherlands is a text book example of a country with a manure surplus problem. As a dairy, egg and meat exporting country, it has one of the highest livestock density in Europe and imports most of its feed (van Selm *et al*. 2023, Eurostat 2024). To provide a numerical perspective, the Netherlands had 11 million pigs and 93 million chickens in 2023, producing approximately 9.7 million tons of manure (CLO 2024a, 2024b). Manure availability far exceeds its use as fertilizer and most soils and surface waters are polluted with nitrate and phosphate, predominantly caused by agricultural practices (Fraters *et al*. 2016).

Methods for managing surplus manure exist, such as exporting dried manure, incineration, aerobic composting, anaerobic digestion and advanced chemical separation techniques (Petersen *et al*. 2007, Leenstra *et al*. 2014). However, these methods either provide little added value or result in simple end products such as salts, methane and carbon dioxide that are in the current financial system, not cost-effective. A more effective approach would mimic nature's bioconversion processes, using organisms like insects or worms to convert nutrients in manure into macromolecules (proteins, fats, chitin) that can be used as livestock feed ingredient or for other purposes. This process not only enhances resource use but also accelerates manure decomposition and generates a desirable microbial community, that decreases formation of noxious compounds (ammonia, sulphides) and reduces bacterial pathogen load (Atiyeh *et al*. 2002, Awasthi *et al*. 2020, Abd Manan *et al*. 2024).

In the class Insecta, the orders Coleoptera (beetles) and Diptera (flies) contain hundreds of species adapted to using digestible fractions of animal manures for at least part of their life cycle (Perri *et al*. 2023). The ideal insect for bioconversion of manure couples a high nutritional value and a low feed conversion rate with a high reproduction rate (Makkar *et al*. 2014, Oonincx 2015, Hussein *et al*. 2017). Also, its physical and behavioral demands need to be relatively simple in order to perform in artificial mass-rearing systems.

The housefly (*Musca domestica* (L.): Diptera: Muscidae) fits several of the criteria. It combines a high nutrient content with a relatively easy husbandry, high fecundity, a short life cycle and tolerance to crowding. Housefly larvae can live on a range of decaying organic materials including animal manures. The industrial mass-rearing potential of housefly larvae has already been demonstrated by successful pilot projects (Čičková *et al*. 2012, Wang *et al*. 2013, Anene *et al*. 2013, Makkar *et al*. 2014, Hussein *et al*. 2017, Miranda *et al*. 2023). Meal made from housefly larvae (maggot meal) varies in crude protein content between 40 to 60% in the dry matter (DM), while the fat content has been found to vary from 9 to 20% DM (Zuidhof *et al*. 2003, Pretorius 2011, Makkar *et al*. 2014, Boatta *et al*. 2023). Larval protein has a balanced amino acid profile resembling that of fish meal, with high amounts of lysine, threonine, methionine and cysteine (Gadzama and Ndudim 2019, Hashizume *et al*. 2019). Maggot meal has shown potential as a partial substitute for fish and soybean meals in poultry diets (Hwangbo *et al*. 2008, Pieterse *et al*. 2014, Sanchez-Muros *et al*. 2014, Khan *et al*. 2016). It may also replace fish meal in the diets of cultured fish, crustaceans, and bullfrogs, with complete substitution limited by an insufficient n-3 and n-6 fatty acid content (Fashina-Bombata and Balogun 1997, Fasakin *et al*. 2003, Ajani *et al*. 2004, Aniebo *et al*. 2008, Zheng *et al*. 2010, Li *et al*. 2019).

Manure bioconversion by houseflies at different scales has been studied over the years (Table 1). In these studies, bioconversion is often expressed on an as-is basis. Because of potentially large differences in moisture content, this unit of measurement is less suitable for cross-study comparisons, whereas expression on a DM- and N-basis provides more reliable comparisons (Bosch *et al*. 2019). As only one study reported a conversion based on DM, all other data in Table 1 were converted to a DM based for comparison. Data on N were not provided, making it impossible to obtain accurate N conversion efficiency values. The variation between experiments in DM conversion is large (0.06 to 18%), which is in line with bioconversion of manures by black soldier fly larvae (3 to 12% DM) (Bosch *et al*. 2019).

Table 1. Manure type, manure moisture, temperature, density (larvae/g manure as-is), harvest time, harvest type (DP: dried pupae, FP: fresh pupae, FL: fresh larvae), survival (% of eggs grown to harvestable larvae or pupae), bioconversion efficiency of fresh matter (BE_{FM}, % of harvested substrate/fresh substrate), bioconversion efficiency of dry matter (BE_{DM}, % of dry harvested substrate/dry fresh substrate) and reduction efficiency (RE_{FM}, % of reduction of fresh matter).

	Manure				Harvest		Larval performance			
Type	**Mass (kg)**	**Moisture (%)**	**Temp (°C)**	**Density (#/g)**	**Time (days)**	**Type**	**Survival (%)**	**BE_{FM} (%)**	**BE_{DM} (%)**	**RE_{FM} (%)**	**Source**
Pig fresh	5	80-85	25	11.0	9	DP	61	na	na	82	Čičková *et al.* 2012
Pig aged sawdust	5	70-80	25	4.4	7	DP	73	4.4	4.8	35	
Pig aged no sawdust	5	65-85	25	11.0	11	DP	47	7.4	8.1	73	
Pig centrifuged slurry	5	73	25	11.0	8	DP	77	na	na	64	
Pig fresh	0.05	50	30	1	na	FP	54	0.7[4]	na	na	Farkas *et al.* 1998
Pig fresh	0.05	60	30	1	na	FP	51	0.5[4]	na	na	
Pig fresh	0.05	70	30	1	na	FP	59	1.4[4]	na	na	
Pig fresh	0.05	80	30	1	na	FP	60	1.3[4]	na	na	
Pig fresh	1000	78	28-42	4	7	FP	na	11.3[1,2,3]	15.0[1,2,3]	60[1]	Zhang *et al.* 2012
Pig	10000	76	na	10	4	FL	na	na	17.6	na	Yang *et al.* 2014
Laying hen fresh	0.1	na	26	1.0	na	FP	98	1.8[3,4,5]	1.6[3,4,5]	80	Barnard *et al.* 1998b
Laying hen fresh	0.1	na	26	3.0	na	FP	86	2.6[3,4,5]	2.4[3,4,5]	64	
Laying hen fresh	0.1	na	26	6.0	na	FP	25	1.1[3,4,5]	0.9[3,4,5]	80	
Laying hen fresh	0.1	na	26	9.0	na	FP	1	0.4[3,4,5]	0.4[3,4,5]	60	
Chicken fresh	0.05	50	30	1	na	FP	54	1.0[4]	na	na	Farkas *et al.* 1998
Chicken fresh	0.05	60	30	1	na	FP	51	1.0[4]	na	na	
Chicken fresh	0.05	70	30	1	na	FP	55	1.3[4]	na	na	
Chicken fresh	0.05	80	30	1	na	FP	51	1.3[4]	na	na	
Chicken fresh	1	73	28	1.0	4	FL	90	2.2	2.0[3]	na	Cai *et al.* 2017
Chicken fresh	1	73	28	2.0	4	FL	87	4.8	4.3[3]	na	
Chicken fresh	1	73	28	3.0	4	FL	85	6.5	5.9[3]	na	
Chicken fresh	1	73	28	4.0	4	FL	82	8.3	7.5[3]	na	
Chicken fresh	1	73	28	6.0	4	FL	81	8.8	7.9[3]	na	
Chicken fresh	1	73	28	8.0	4	FL	80	10.2	9.2[3]	na	

Chicken fresh	1	73	28	10.0	4	FL	70	9.5	8.6[3]	na	
Chicken fresh	1	73	28	12.0	4	FL	55	7	6.3[3]	na	
Laying hen fresh	0.06	75	26	1.7	8	FP	73	3.2[4]	na	na	Miranda *et al.* 2019a
Laying hen fresh	4	75	27	1.4	8	FP	na	na	2.6	na	Miller *et al.* 1974
Laying hen fresh	4	75	27	2.8	8	FP	na	na	5.0	na	
Laying hen fresh	4	75	27	4.2	8	FP	na	na	7.6	na	
Laying hen fresh	4	75	27	5.6	8	FP	na	na	7.1	55	
Laying hen fresh	4	75	27	6.3	8	FP	na	na	2.4	na	
Chicken fresh	0.2	na	27	1.5	8	FP	85	1.1	1.0[3,5]	49	Calvert *et al.* 1970
Chicken fresh	0.2	na	27	2.0	8	FP	66	1.4	1.2[3,5]	56	
Chicken fresh	0.2	na	27	3.0	8	FP	44	1.6	1.5[3,5]	58	
Laying hen fresh	4	na	25	0.9	8	DP	na	2.7[3,5]	2.4	40	Miller *et al.* 1974
Laying hen fresh	4	na	25	1.9	8	DP	na	5.1[3,5]	4.6	46	
Laying hen fresh	4	na	25	2.8	8	DP	na	7.8[3,5]	7.0	51	
Laying hen fresh	4	na	25	3.7	8	DP	na	7.3[3,5]	6.6	46	
Laying hen fresh	4	na	25	4.2	8	DP	na	2.5[3,5]	2.2	44	
Dairy cow fresh	0.025	85	28	1.0	5	FL	79	0.4	0.06	5	Hussein *et al.* 2017
Dairy cow fresh	0.025	85	28	2.0	5	FL	75	0.6	0.10	11	
Dairy cow fresh	0.025	85	28	4.0	5	FL	70	0.9	0.15	21	
Dairy cow fresh	0.025	85	28	8.0	5	FL	71	1.4	0.23	25	
Dairy cow fresh	0.025	85	28	16.0	5	FL	63	1.8	0.31	27	
Cow	0.05	78	27-30	1.7	10	FP	83.3	0.6	na	na	Larraín and Salas 2008
Calf	0.05	69	27-30	1.7	10	FP	89.2	0.9	na	na	
Swine	0.05	66	27-30	1.7	10	FP	97.5	1.2	na	na	
Chicken	0.05	65	27-30	1.7	10	FP	95	1.1	na	na	
Chicken	2	70	24-32	7	5	FL	na	na	4.1	na	Ganda *et al.* 2021

na = not available.[1] Averages calculated based on the range of wet manure given. [2] Fresh larval mass estimated from a graph.[3] BE_{DM} calculated with a larval dry matter content of 24.3% based on Hussein et al. (2017). [4] Fresh larval mass calculated from survival and mean pupal mass given. [5] Manure dry matter of 73% assumed from Cai *et al*. (2017). [6] Egg densities derived from g/kg and weight of single egg = 0.07 mg.

The variation in bioconversion expressed on an as-is and DM basis can be related to differences in experimental procedures, such as the scale (25 g to 1000 kg), the different harvest times of larvae and incubation at different temperatures. This large variation does not allow for generalizations regarding how yields are related to animal type or harvest time. Most publications involve chicken manure, with only one study using cow manure in very small amounts (25 g). The limited data do provide some insights from a larvae-rearing perspective.For chicken manure, larger amounts of manure seem to improve bioconversion efficiencies which are two to four times higher when larger quantities (1 and 4 kg) are used in comparison to when smaller quantities (0.1 and 0.2 kg) are used. This is likely explained by multiple factors, such as lower susceptibility of larger quantities to drying or temperature variations and the positive effects of a larger larval mass. Furthermore, higher densities of larvae stir the substrate and aerate the manure more, potentially improving the conditions for beneficial microorganisms (see nutritional ecology section below).

Challenges for housefly larvae mass-rearing

Although mass-rearing of housefly larvae for use as animal feed and waste management has a high potential, there are several challenges to turn it into a viable economic endeavour. The challenges fall broadly into three categories, regulatory, economic and operational.

Regulatory challenges predominantly concern transmission of pathogens and contaminants such as mycotoxins and veterinary medicines. Currently, insects that have been grown on animal manure are not allowed to enter the human food chain in the European Union (Baiano 2020, Delgado *et al*. 2023). Concerns about zoonotic pathogens are legitimate as houseflies are notorious vectors of over a hundred species of pathogens and parasites, from viruses and bacteria to protozoa and helminths (Liu *et al*. 2023). Of main concern are species acquired by larvae from manure that can persist through the larval instars, such as *Escherichia*, *Clostridium*, *Listeria*, *Salmonella*, *Streptococcus* and *Pseudomonas* spp. (Kobayashi *et al*. 1999, Omalu *et al*. 2009, Pava-Ripoll *et al*. 2015). Studies on the persistence of *Escherichia coli* strains in larvae indicate that *E. coli* is acquired from manure but that presence in late instars depends greatly on the prevalence in manure and digestion of the bacteria (Rochon *et al*. 2004, 2005, Schuster *et al*. 2013). Mitigating transmission of manure pathogens and toxins or removing these from larvae will be an important subject for near-future research. As food borne zoonoses and toxins are nothing new, existing surveillance and prevention methods can be tested and adapted for manure grown housefly larvae. For example, starving or heating larvae could be part of the harvesting process or manure could be pasteurized or otherwise treated before used as larval substrate.

Associated with regulatory challenges are economic challenges. Industrial mass-rearing of housefly larvae will only be profitable if market demand is stable and regulations are conducive to commercial operations. For now prohibition is halting regulatory approval of manure-reared insects. Apart from this regulation-derived challenge, there might be different economic challenges such as consumer perception and competition with other insects that are already more established, such as meal worms or black soldier flies reared on vegetable waste streams.

Operational challenges involve feasibility and efficiency. Scaling up housefly larvae production requires advancements in mass-rearing technology, including process automation, waste disposal methods and efficient egg collection systems. While pilot projects have been successful, these processes need significant optimization to meet industrial demands (Čičková *et al*. 2015). Additionally, managing disease risks in mass-rearing systems will be critical. Houseflies are a prey or host for other arthropods, nematodes, bacteria and fungi, some of which are associated with animal manures. The dynamics of host-parasite interactions in mass-rearing environments differ from those in natural settings, which can lead to disease outbreaks and make disease management a complex issue (van Baalen and Sabelis 1995). Lessons from other insect industries like beekeeping and sericulture, that have faced severe challenges with parasites and pathogens, can possibly contribute to deriving best practices for housefly rearing (Rozenkranz *et al*. 2010, Bebitha *et al*. 2016).

Knowledge gaps

A key knowledge gap among operational challenges is the limited understanding of larval nutrition. Though larvae grow on manure, little is known about what determines nutritional quality of manure for larvae and how this relates to larval growth and production of larval biomass. Manure is a very transformative material, with rapidly changing (bio)chemistry affecting nutrient availability (see section nutritional ecology below). For example, the discussion about the bacterial contribution to the larval diet is largely open, whether larvae ingest and digest specific bacterial taxa and if these are ingested alive or as dead bacterial biomass is not known. And it is also unknown whether manure can be treated, and if so how, to improve nutrient availability to optimize bioconversion and larval biomass production. This thesis addresses a small part of this knowledge gap, focusing on improving larval growth on and bioconversion of chicken manure for housefly mass rearing.

1.2 Nutritional physiology, phylogeny and ecology of housefly larvae

To have a better understanding of how housefly larvae utilize nutrients from manure, the following sections summarize current knowledge on larval digestive physiology and nutritional requirements, combined with perspectives from housefly phylogeny and nutritional ecology.

Larval digestive physiology

The digestive tract of both adult and larva consists of a pharynx, oesophagus, proventriculus, midgut (anterior, middle and posterior) and a hindgut that includes the rectum. The oesophagus is also connected to the crop, close to the proventriculus and to the salivary glands, close to the pharynx. The posterior part of the midgut is encompassed by the Malpighian tubes that have a function in nitrogenous waste transport and osmoregulation. They merge with the hindgut to secrete pre-urine which combines with undigested food to form the feces. The division in functional parts of the larval digestive tract is similar to that of the adult but the larval tract is much longer, possibly to provide the large surface area needed for a high uptake of nutrients that enables the fast growth during this life stage (Hewitt 1910). A notable difference between adult and larval digestive tract morphology is the structure of the pharynx which contains pharyngeal ridges, a structure of channels and lamellae at the bottom of the pharynx. These lamellae act as a sieve for particles when muscular action forces liquid through the lamellae. The housefly shares this feature with other saprophytic muscoid fly species and it is considered an essential adaption to a diet largely composed of microorganisms (Dowding 1967, Lemos and Terra 1991, Nayduch and Burrus 2017).

Dowding (1967), often cited as proof that housefly larvae ingest bacteria, focused on the structure of the pharynx of third (last) instar larvae as this could be studied with available microscopic technique. She had probably no idea that the external mouth parts undergo drastic changes between the first and the last instar, only observable by electron microscopy. The first instar mouth parts consist of a triangular field of small sclerotized cirri around a central oral ridge that leads to the oral opening, similar to the mouthparts of a snail. The third instar mouthparts consist of ridges without cirri that lead to the oral opening, with both mandibular sclerites ending in a protruding hook structure (Skidmore 1985, Szpila and Pape 2008, Cortinhas *et al*. 2020). This change in mouth parts morphology might indicate ingestion of different materials by different larval instars (see subsection on nutritional ecology below).

Moving from digestive morphology to digestive enzymes, Greenberg and Paretsky (1955) were among the first to describe that proteolytic activity in the larval gut of the housefly took place at a pH of 2.5 to 3.0 and suggested that the responsible protease was a pepsin. Subsequent work confirmed that proteolysis occurs in the midgut at low pH (~3) and that the enzyme previously reported as pepsin is instead a low-pH-active aspartic protease (cathepsin) (Greenberg and Paretsky 1955, Lemos and Terra 1991).

Consistent with reports of trypsin-like proteases, a trypsin was found only inside cells rather than in the lumen (Jordão *et al*. 1996). Proteolytic activity in all three larval stages was predominantly due to aminopeptidase-like enzymes and much less (15 fold) due to trypsin- and chemotrypsin-like

enzymes (Blahovec *et al*. 2006). Also, the concentrations of aminopeptidase-like enzymes was roughly fourfold higher in first instar larvae compared to second instar and roughly tenfold compared to the third instar. This could indicate that younger larvae are more dependent on protein and possible consume a different diet than older larvae (see subsection nutritional ecology below).

Cellulase and beta-glucosidase were identified in the salivary glands of housefly larvae, suggesting they can digest cellulose (Hu *et al*. 2013). However, this finding lacks confirmation from other studies. In addition, trehalase, amylase and invertase were found in larval gut extracts (Hirashima *et al*. 1989). Different amylases were found in larvae, indicating flexibility in starch digestion (Terra and Jordão 1989, Pimentel *et al*. 2018). Based on gene expression of enzymes and transporter molecules, Pimentel *et al*. (2018) propose a digestion pathway. Starch is broken down by amylases and maltases in the anterior midgut, followed by uptake of the resulting glucose units in the middle midgut. Although digestive lipases were isolated, hydrolysis of fats and oils by housefly lipases was reported to be very low (Baker and Paretsky 1958, Ahmad 1970, De Bianchi *et al*. 1987).

Lysozyme hydrolyzes peptidoglycans, a key component of bacterial cell walls, and serves as part of the innate immune response across diverse taxa (Prager and Jollès 1996, Callewaert and Michiels 2010). However, in housefly larvae, adapted to a microorganism-rich diet, lysozyme functions as a digestive enzyme (Lemos and Terra 1991, Nayduch and Joyner 2014, Pimentel *et al*. 2018). Ito *et al*. (1995) found that housefly lysozyme closely resembles ruminant digestive lysozymes rather than vertebrate immune lysozymes that have a lower molecular weight and are active at lower pH. In ruminants, these enzymes primarily target gram-positive Firmicutes, such as *Ruminococcus* and *Clostridium* spp.(Russell and Rychlik 2001, Henderson *et al*. 2015). Beyond digestive lysozyme, housefly larvae produce various antimicrobial compounds, predominantly peptides and proteins, mostly targeting gram-positive bacteria (Niode *et al*. 2022). Their role in digestion remains unclear, as studies have used whole-larvae extractions or gene sequences (Moreira *et al*. 2003, Fu *et al*. 2009, Zhou *et al*. 2014, Wang *et al*. 2022). If larvae secrete antimicrobials into the substrate and absorb lysed bacterial contents, this would suggest an “external stomach” effect, but this has yet to be investigated.

Larval nutritional needs

The housefly has been extensively studied due to its role in pathogen transmission and insecticide resistance, which has driven the development of laboratory diets. Additionally, the housefly was a popular early model species to unravel insect nutritional needs.

Chang and Wang (1958) demonstrated that sterilized housefly eggs could develop into adults on a synthetic, sterile medium. This medium contained agar, casein, dextrose, ribonucleic acid, cholesterol, the vitamins A, C, D and the B-vitamins thiamine, riboflavin, niacin amide, and pyridoxine hydrochloride. It also contained the minerals calcium phosphate, calcium lactate ferric citrate magnesium sulphate potassium phosphate, sodium phosphate and sodium chloride. Successful development of larvae on this medium indicated that microorganisms were not necessary. However, omitting ribonucleic acid, vitamin A, C or D led to retarded development. Also, no larval growth was

observed when the casein was replaced by casein hydrochloride hydrolysate. The authors contributed this to the destruction of tryptophan during hydrolysis. Substitution of casein by amino acid mixtures showed that arginine, lysine, leucine, isoleucine, tryptophan, histidine, phenylalanine, methionine, valine and threonine are essential for larval development. Perry and Miller (1965) confirmed that folic acid is also critical for larval growth. This aligns with the necessity of B vitamins and ribonucleic acid in larval diets (Brookes and Frankel 1958).

The need for lipids in the larval diet has been studied (Bergmann and Levinson 1954, Silverman and Levinson 1954, Monroe 1959, Dwivedy 1975). All these authors concluded that housefly larvae have no need for fatty acids but that a sterol is essential. Housefly larvae were found able to use the phytosterols campesterol, stigmasterol and sitosterol, the mycosterol ergosterol and the zoosterols cholesterol, coprostanol and dehydrocholesterol (Kaplanis *et al*. 1960, Spiller 1964, Stevanov *et al*. 2002). Insects have lost the capability for sterol biosynthesis (Fügi *et al*. 2014). Biosynthesis of sterols is metabolically costly and reliance on external sterols seems an adaptation to specific ecological niches that have sufficient sterol supply, indicating early coevolution with certain plants or fungi (Jing and Behmer 2020). The vast majority of bacteria, Proteobacteria, Firmicutes and Actinobacteria, do not synthesise sterols. As such it is logical to assume that the source of sterol for housefly larvae originates from plants, fungi or animal material. In the case of manure, this contains undigested dietary components, endogenous losses of the host and yeasts growing in the gut (*Candida* spp.) or grow post-defecation (Schulze and Sonnenborn 2009).

Brookes and Frankel (1958) showed that larvae do not require starch, simple carbohydrates or lipids as these either had no effect or inhibited growth. The authors make two interesting observations regarding the development of their basic diet: brewer's yeast could replace the need for ribonucleic acids and all the B vitamins, but fractions of brewer's yeast could not. And in all semi-synthetic sterile media, larval growth was 10 to 15% slower and final larval weight 30 to 40% lower compared to non-sterile CSMA (Chemical Specialties Manufacturers' Association) larval medium. The CSMA medium consists of alfalfa meal, spent brewer's grains and wheat bran and is derived from the experiments of Richardson (1932) who was investigating to substitute horse manure. Richardson's original medium contained wheat bran, alfalfa meal, yeast and malt. Evaluation of laboratory diets led to the development of the Gainesville Housefly Diet, with brewer's grains and yeast omitted and corn meal added (Hogsette 1992).

Although housefly larvae do not need carbohydrates, they grow well on the high starch CSMA and Gainesville diets. However, sterilizing these diets appears to significantly hinder growth. Greenberg (1954) noted deficient larval development on sterilized wheat bran substrates unless it was pre-incubated, suggesting that microorganisms or their byproducts were critical and the latter were not destroyed by heat sterilization. Greenberg did not speculate on which byproducts were critical but assuming adequate protein (bio)availability, the supposed compounds could be heat stable B vitamins (B3, B5, B7) or bacterially modified sterol. Wheat bran is rich in phytosterols but these might not all be useable by larvae. By incubating wheat bran and milk powder media inoculated with *E. coli*, the suspicion of Greenberg was empirically tested by Levinson (1960). Larval growth was strongly

associated with the number of *E. coli* present in the medium, and above a threshold, larval growth and survival were comparable to natural media. Feeding experiments using fractions of the substrate consolidated that bacteria were the key factor for larval nutrition and that the bran medium was a substrate for bacterial growth and not a nutrient itself. Inoculating sterile blood agar with different bacteria again confirmed that microorganisms are essential for larval growth while suggesting that bacteria primarily provide B vitamins rather than protein (Schmidtmann and Martin 1992).

In addition to nutrients, other characteristics of substrates are important for larval growth. Housefly larvae tolerate a temperature range of 13-38 °C, with optimal growth occurring between 20-30 °C (Lysyk and Axtell 1987, Barnard and Geden 1993). Moon *et al*. (2001) showed that larvae could survive and burrow in sand, loam and sandy loam, provided the moisture content was within the optimal range (60-70%). This moisture range was also identified as crucial for larval growth in poultry manure (Fatchurochim *et al*. 1988, Farkas *et al*. 1998).

Nutritional insights from housefly phylogeny

Phylogenetic comparisons contextualize housefly biology within evolution and offer insights into larval diets and physiology that may not be directly observable. Following Yeates *et al*. (2007), *Musca domestica* belongs to the order Diptera, suborder Brachycera, infraorder Muscomorpha (syn. Cyclorrhapha). Within Muscomorpha, it is placed in the calyptrate Schizophora, superfamily Muscoidea, family Muscidae, subfamily Muscinae and genus *Musca*.

Besides the Muscoidea, the calyptrate Schizophora contains the superfamilies Oestroidea (flesh and carrion flies) and Hippoboscoidea (bat and tsetse flies). The carnivorous or necrophagous larvae of most Oestroidea species live in/on animal tissue (causing myasis), while Hippoboscoidea larvae are retained in the mother and feed on an uterine secretion (Maślanko *et al*. 2022). The widespread carnivory and necrophagy of larval Schizophora raises the question if larval digestion capabilities of *Musca* are derived from a carnivorous or necrophagous ancestor. Especially concerning its (facultative) starch digestion, with amylase also able to break down glycogen, potentially present in (dead) animal tissue (Terra and Ferreira 1994).

This does not appear to be the case. Genes for different α-amylases are widely distributed across the Muscoidea, having originated in a duplication event ancestral to Schizophora (Maczkowiak and Lage 2006). *Musca domestica* exhibits high diversity in amylase genes, Black and Krafsur (1985) identified five different copies in adult flies, producing functionally distinct amylases varying in pH sensitivity and amylose affinity. Terra *et al*. (1988) further demonstrated that different genes encode larval and adult amylases, highlighting life stage-specific expression. Compared to *Drosophila melanogaster*, *Musca domestica* possesses greater amylase diversity, which aligns with the idea that Muscoidea evolved to exploit plant-derived saprophytic diets (McCommas and Shornick 1990). Consequently, starch digestion in *Musca domestica* larvae is not a novel adaptation but an ancestral trait dating back at least 42 million years (Ding *et al*. 2015). Thus, the carnivorous and parasitic diets of Oestroidea larvae represent a more recent evolutionary divergence from this ancestral state.

The subfamily Muscinae, with mostly saprophagous larvae, is divided into two families. The Muscini, which include the genus *Musca*, and, as nature loves to evolve hematophagous adult stages independently across the Diptera, the Stomoxini, which include the genera *Stomoxys* and *Haematobia*. Nearly all *Stomoxys* and *Haematobia* larvae are highly specialized in using fresh manures (Skidmore 1985)

The genus *Musca* has undergone drastic taxonomical revisions as multiple species look extremely similar. It currently contains approximately 50 species (Yeates *et al*. 2007, King 2019). Of these, *Musca domestica* larvae appear the most dietary flexible, exhibiting necrophagy, saprophagy and coprophagy. Some *Musca* species larvae are obligate coprophages, e.g. *Musca sorbens* larvae specialized in human feces (Emerson *et al*. 200). The high adult and larval dietary flexibility makes *Musca domestica* highly suited for a synanthropic lifestyle, with human hunter-gatherer and agricultural societies providing abundant feed materials. Both humans and *Musca domestica* appear to have evolved in Africa before spreading across the globe together (Haseyama *et al*. 2015).

The genome of *Musca domestica* has been sequenced (Scott *et al*. 2014). Compared to *Drosophila melanogaster*, an acalyptrate Schizophora, *Musca domestica* has a much higher genetic diversity for immune proteins and detoxification enzymes, reflecting its high exposure to bacteria and noxious compounds in decaying materials. Interestingly, the genome contains many duplicates and novelties concerning chemoreception and gustation, meaning that *Musca domestica* has a highly developed sense of smell and taste. Scott *et al*. (2014) mention that genes that code for receptors involved in tasting bitter substances are highly abundant and also attribute this to exposure to decaying materials. These authors state that the ligands for bitter receptors are unknown. In nature, two families of molecules tend to be bitter tasting, plant metabolites and certain peptides and amino acids (e.g. l-phenylalanine, l-tyrosine) resulting from protein hydrolysis (Chu *et al*. 2014). That houseflies avoid insecticidal plant metabolites is apparent but, speculating, the avoidance of specific peptides or amino acids might be a way of female houseflies to assess protein quality for larvae (see section nutritional ecology below).

Phylogenetic studies also provide a perspective to compare *Musca domestica* and another promising candidate for manure bioconversion, *Hermetia illucens*, the black soldier fly (Box 1).

Box 1. *Musca domestica* versus *Hermetia illucens*

Though *Musca domestica* and *Hermetia illucens*, the black soldier fly, both belong to dipteran suborder Brachycera, their phylogeny diverges significantly. *Musca* is classified within the Muscomorpha superfamily Muscoidea, while *Hermetia* belongs to the Stratiomyomorpha superfamily Stratiomyoidea. The Stratiomyoidea are substantially older (80-100 million years) than the more recently evolved Muscoidea (Wiegmann *et al*. 2000).

The Stratiomyoidea contains three families, Pantophthalmidae, Xylomyidae and Stratiomyidae (soldier flies). The larvae of most species in the first two families are xylophagous (wood eating), which is rather unusual in the *Diptera* and involves highly specific relations with microbiota (Ulyshen 2016). The Stratiomyidae subfamily Hermetiinae contains the genus *Hermetia*, consisting largely of wasp mimics. This includes *Hermetia illucens*, which resembles a potter wasp (Eumeninae) rather than a muscoid fly.

Adult *Hermetia illucens* are substantially bigger than *Musca domestica* (~15 mm) and much shorter lived. The larvae are big (~15 to 20 mm) and have a much longer larval stage (18 to 36 days) than *Musca domestica* (Tomberlin *et al*. 2002). The saprophagous larvae can grow on a variety of diets with a high microbial load. The structure of the larval gut and activity of digestive enzymes has been well studied as *Hermetia illucens* is already in use for mass-rearing on plant based diets (Bonelli *et al*. 2019, Intayung *et al*. 2021). *Hermetia illucens* larvae supposedly live on a wider variety of diets than *Musca domestica* because cellulases and chitinases are in its enzymatic repertoire (Intayung *et al*. 2021). Unlike *Musca domestica*, *Hermetia illucens* adults are mainly nectar feeders, reducing their potential for pathogen transmission, though pathogen and toxin transmission from substrates to larvae does occur (Bessa *et al*. 2021).

To those who have rearing experience with the two species, adult behaviour is strikingly different. In comparison to fast and agile adult *Musca*, adult *Hermetia* are almost immobile and hardly able to navigate indoor spaces.

Nutritional ecology of housefly larvae in manure

Nutritional ecology, that examines the relationships between diet, behavior and environmental conditions, is highly informative to aid understanding of how housefly larvae utilize manure. Unlike pigs and poultry, for whom feed conversion relies on enzymatic digestion and microbial interactions within their digestive tracts, housefly larvae are surrounded by manure that is highly transformative due to environmental and microbial interactions. This section briefly explores (potential) relations between manure, larvae and microorganisms to identify strategies for optimizing larval growth.

Manure suitable as larval substrate must be fresh. Fresh manure concerns feces or excreta (bird feces mixed with uric acid) voided less than 24-36 hours. Aged manures are not attractive for houseflies and larval growth is low or absent (Miller *et al*. 1974, Barnard *et al*. 1998, Broce and Haas 1999, Moon *et al*. 2001, personal observation). Fresh manure consists of bacterial biomass, undigested dietary components, endogenous losses (enzymes, epithelial cells, mucins) and water.

Table 2 summarizes the macronutrient content of fresh manure from the main livestock species with horse manure included for comparison, as it was historically used as a larval substrate. The number of studies is limited and variation exists within species but some generalizations can be made regarding the nutritional properties of manure for larval diets.

Table 2. Range of macronutrient and fiber composition (% of dry matter) of fresh cow, pig, chicken (broiler and layer combined) and horse manures.

Animal	%DM	Total N	Crude fat	Hemi-cellulose	Cellulose	Lignin	Source
Horse	20.7-27.3	1.2-1.9	1.8-4.1	-	-	-	Mönch-Tegeder *et al*. 2014
Cow	12.6-21.5	1.9-2.9	-	12.2-21.4	14.2-27.4	6.1-13.0	Chen *et al*. 2003
Cow	11.8-15.6	1.4-2.6	3.7-12.3	11.4-26.6	19.6-25.9	13.4-19.2	Moller *et al*. 2014
Cow	13.4-18.5	-	-	-	-	-	Van Vliet *et al*. 2007
Cow	18.6	1.1	4.0	-	-	-	Chinwendu *et al*. 2013
Cow	15.1	2.4	1.2	-	-	-	Hussein *et al*. 2017
Pig	-	3.25-4.1	-	20.4-21.9	13.2-13.9	4.1-6.4	Chen *et al*. 2003
Pig	18.0-27.2	1.8-5.0	3.5-5.9	18.8-26.2	9.8-22.9	2.9-6.0	Hilliard *et al*. 1979
Chicken	-	4.5-7.7	-	16.4-21.5	7.7-12.0	2.3-7.2	Chen *et al*. 2003
Chicken	72.7-89.1	2.3-6.4	0.8-3.5	-	-	-	Martin *et al*. 1983

Cows digest low-protein (commonly 5 to 15% DM), fiber-rich diets utilizing their rumen microbiota, primarily consuming fresh grass, maize, wheat roughage or silages, often supplemented with soy, alfalfa or rapeseed cake to boost protein intake for milk production (Moller *et al*. 2014, Schwab and Broderick 2017, Rauch *et al*. 2021). Consequently, fresh cow feces are low in nitrogen but high in undigested fractions of the consumed fibrous feedstuffs (Chen *et al*. 2003, 2005, Van Vliet *et al*. 2007). Pigs, monogastric omnivores, require more protein and starch and are typically fed cereal meal, rapeseed meal, soy concentrates and beet pulp containing roughly 20% protein (Aarnink and Verstegen 2007, De Lange *et al*. 2010, Blok *et al*. 2015). Their feces are higher in nitrogen and lower in fiber than cow feces. Chickens, also monogastric omnivores, consume similar diets with similar protein content, composed of cereals (corn, wheat, sorghum, rice bran) and protein sources like soy meal, fish meal and rapeseed meal (Leeson and Summers 2008). Their excreta (feces + urine) have nitrogen levels comparable to or higher than pig feces, with a high hemicellulose fraction. As birds excrete excess nitrogen as uric acid, non-protein nitrogen in chicken excreta ranges from 16 to 60% (Koerkamp 1994, El-Deek *et al*. 2009, Apajalathi and Vienola 2016). Though larvae have been reared on cow manure, pig and chicken manures appear to be better substrates, leading to larger (heavier) larvae and lower egg-pupae or egg-adult mortality, with better growth related to higher protein content (Larraín and Salas 2008, Ganda *et al*. 2021, Kakuru *et al*. 2022, Miranda *et al*. 2023).

Fresh manure of aforementioned animals contains little to no digestible carbohydrates, as most starches and sugars are digested and absorbed in the intestine, with remaining material fermented in the colon (Bender 2014, Svihus 2014).

Fecal protein primarily originates from bacteria, which make up 10-30% of faecal DM, with bacterial DM consisting of 50-80% protein (Levinson 1960, Nodar *et al*. 1990, Kurbanoglu and Algur 2002,

Ritala *et al*. 2017). The fecal mixture of bacterial and endogenous protein contains all the amino acids essential for housefly larvae, with aspartic and glutamic acid being most abundant (Chen *et al*. 2003).

All fresh manures contain sterols but their content varies depending of the species and individual manures originate from, predominantly depending on dietary sterol content and chemical modification by gut microbiota (Leeming *et al*. 1996, De Smet *et al*. 1998). In pig manure, the main sterols tends to be coprostanol and epicoprostanol, whereas in chicken excreta cholesterol, 24-ethylcholesterol and sitosterol are the most common (Loughrin and Szogi 2006, Derrien *et al*. 2011, Devane *et al*. 2015). Additionally, feces contain volatile fatty acids (e.g. acetic and butyric acid) and nitrogenous fermentation byproducts (e.g. putrescine, skatole), that can rapidly evaporate upon exposure to air (Charalampopoulos *et al*. 2002, Seravalli and Portugal 2023).

In the remainder of this section, housefly larval ecology is described specifically for fresh chicken manure, for reasons elaborated in the section “thesis aim and theoretical approach”.

After defecation, fresh chicken manure undergoes a dramatic environmental change, as it is suddenly exposed to oxygen, dehydration and lower temperatures. This change drives a major transformation in diversity and abundance of fecal microbiota during the first 72 hours (Oladele *et al*. 2024). All obligate anaerobe Firmicutes (mainly gram-positive Clostridia and Bacilli, e.g. *Ruminococcus*) that make up most of the microbial biomass, die off, partly releasing their proteinaceous cell contents into the manure biomass. Concurrently, aerobe Proteobacteria and Bacteroidota (e.g. the gram-negative genera *Escherichia* and *Shigella*) increase in both diversity and biomass. Some facultative anaerobe or aerotolerant Actinobacteria (e.g. the genera *Bifidobacterium*, *Corynebacterium*, *Actinomyces*) continue to increase in abundance after 48 to 72 hours (Klug *et al*. 1998, Yang *et al*. 2021, Oladele *et al*. 2024). Actinobacteria are prime decomposers of recalcitrant plant polymers, able to grow on fiber fractions of manure (Lewin *et al*. 2016). This gram-positive bacterial group might be a target for larval lysozyme and a potential important food source for (older) larvae. Several Actinobacteria are capable of producing glycogen (Barka *et al*. 2015), that as mentioned before can be a substrate for larval amylases.

After the die off of the anaerobic Firmicutes, fresh chicken manure has a high protein content, a high uric acid content and a very low digestible carbohydrate content. Microbial enzymatic activity is predominantly proteolytic, with the growing population of Proteobacteria and some facultative anaerobe Firmicutes emitting different proteases into the manure biomass, while to a lesser extent Bacteroidota species emit different cellulase type enzymes that digest non-starch polysaccharides present (Zhang *et al*. 2018, Zainudin *et al*. 2022). This temporal high protein availability coincides with the high protease activity of first instar larvae compared to that of later instars (see section larval digestive physiology above).

Interestingly, the abundance of a distinct gram-negative group containing species in the genera *Proteus*, *Pseudomonas* and *Klebsiella* (the “*Proteus* group”, Box 2) also increases rapidly after 24 hours of aerobic exposure (Oladele *et al*. 2024). The *Proteus* group does not participate in the dominant Proteobacteria protein catabolism but metabolizes inorganic nitrogen, emitting ureases that

convert uric acid and urea to ammonia for synthesis of glutamate while using molecules like citrate or organic acids as carbon source (Pearson *et al*. 2011). The generation of ammonia by the *Proteus* group can be substantial and fast, enough to strongly and quickly increase the pH of the manure biomass. Ammonia is toxic to other bacteria and as its ammonium salt is a known inhibitor of some proteases, while activity of other proteases decreases due to increased pH (Liu and Hsieh 1969, Klug *et al*. 1998). Another feature of several *Proteus* group species is the production of anti-fungal metabolites that protected housefly larvae from entomopathogenic fungi in experimental infection (Wang *et al*. 2020).

Box 2. The *Proteus* group

The "*Proteus* group", used here as an informal designation, includes *Proteus* and related genera such as *Morganella, Ignatzschineria, Acinetobacter, Providencia* and *Serratia,* which share similar biochemical and ecological characteristics. Species within this group constitute a major component of the microbiome of houseflies and carrion flies (Oestroidea: Calliphoridae), the first insects to colonize fresh carrion and feces (Junqueira *et al*. 2017). Most belong to the class Gammaproteobacteria, typically within or adjacent to the order Enterobacterales (Adeolu *et al*. 2016).

Apart from their potential positive effects on larval survival and early growth through ammonia production, the *Proteus* group plays a pivotal role in the so-called fly factor, a phenomenon whereby food substrates currently or previously exposed to flies attract further fly aggregation through bacterially produced volatiles. Originally described by Dethier (1955) and later confirmed in several muscoid and calliphorid species (e.g. *Musca domestica*, *Musca autumnalis*, *Phormia regina*, *Lucilia sericata*), the fly factor arises from semiochemicals generated by symbiotic bacteria carried and disseminated by flies (Barnhart and Chadwick 1953, Brodie *et al*. 2015, Holl and Gries 2018, Uriel *et al*. 2022). Among the principal bacterial actors are *Proteus mirabilis, Morganella morganii* and *Ignatzschineria spp.* These bacteria release characteristic odorants, including phenol, indole, methyl-indole, cadaverine and putrescine, through proteolysis and amino decarboxylation of diverse protein substrates. The resulting volatile blend attracts conspecific and heterospecific flies and simultaneously promotes bacterial dispersal. Interestingly, some of these same compounds, notably putrescine, also act as intracellular signals regulating *Proteus* "swarming" behavior, linking chemical communication within and between species (Sturgill and Rather 2004).

Fungal spores make up a large part of the organic biomass in the air (Anees-Hill *et al*. 2022). Fresh manure tends to be quickly colonized by Ascomycetes, for example in the genera *Fusarium* and *Phoma* and by Mucoromycetes of the genera *Rhizopus* and *Mucor*, known as common bread molds (Xie *et al*. 2021). These fungi have in common that they emit proteases to use amino acids as energy source, with many species making anti-bacterial compounds and some emitting chitinases with insecticidal effects (Edgington *et al*. 2014, Muszewska *et al*. 2021). It could be speculated that fungi together with Proteobacteria are the main competitors for housefly larvae in fresh manure.

Whereas fungi depend on coincidence to find substrates, the extremely mobile housefly finds fresh manures by a well-developed olfactory system. Gravid females are strongly attracted by several molecules emitted by fresh manure, such as volatile fatty acids, putrescine, ammonia and indoles (Geden *et al*. 2021, Box 2). If the quality of substrate is adequate, indicated by a "taste test" that

possibly reveals the status of protein availability and microbial activity, females will deposit a clutch of eggs. Oviposition by one female attracts oviposition by other females through release of semiochemicals, leading to mass oviposition of eggs (Jiang *et al*. 2002). Mass oviposition is common in Muscoidea and leads to large local masses of early instar larvae (Johnson and Wallman 2014).

Larval masses benefit from "Allee effects", with individual fitness positively correlated with increased population density (Allee *et al*. 1949). Larval masses are able to raise their temperature above ambient which increases individual metabolism leading to faster development and potentially increased effectivity of digestive enzymes (Rivers *et al*. 2011, Serra *et al*. 2011). Increased enzyme effectivity might be a key component as freshly hatched individual larvae initially have a very limited energy supply, consisting mainly of vitellogenin, a glycolipoprotein that has properties of sugar, fat and protein (McIntyre *et al*. 2000).

In addition to fast substrate detection and mass oviposition, houseflies have two other mechanisms to decrease competition with bacteria and fungi. The first is their symbiotic association with the *Proteus* group (Box 2). The *Proteus* group bacteria are passively acquired during the life of the mother and are transferred from mother to eggs at oviposition (Lam *et al*. 2009a). As the *Proteus* group uses inorganic nitrogen and peptides, they do not compete (much) with larvae for protein. As mentioned, these bacteria quickly increase ammonia levels and possibly emit anti-fungal compounds, which, combined with increased temperature and aeration of larval masses, is a powerful anti-bacterial and anti-fungal strategy. The interaction with the *Proteus* group might particularly benefit first instar larvae that have little energy reserves and time to synthesize anti-microbial compounds. Production of lysozyme and other anti-microbials is the second mechanism, but as this costs protein and energy, it might only be relevant for later larval instars that are able to allocate protein and energy away from growth.

The currently widely accepted model of larval housefly nutrition is that larvae digest bacteria, as postulated, for example, in the often cited article from Nayduch and Burrus (2017). These authors provide a thorough elucidation of proven larval mechanisms for bacterial digestion but do not consider larval ontogeny and bio(chemical) substrate changes. In my opinion, larval digestion must be adapted to temporal nutrient availability, which is strongly related to facilitation by and competition with other organisms in the larval substrate. The substantial changes in mouth part morphology from first to third larval instars and decreased protease activity of later larval instars appear to match temporal protein availability. Possibly, first instar larvae are highly dependent on the more soluble protein from degraded bacterial cells, with only older instars switching to digestion of live or dead bacteria.

1.3 Research justification

The research described in this thesis was funded by the NWO (Netherlands Organization for Scientific Research) initiative "Closed Cycles", that aims innovating resource and waste management to facilitate the transition to a circular economy. Part of this initiative was the project "Towards utilizing livestock manure for mass-rearing of houseflies for feed" (ALW.2016.17), that involves research

conducted by a consortium of the University of Groningen, VU Amsterdam, Wageningen University & Research and the private company Amusca. The latter is a company that produces fresh housefly larvae for the poultry and aquaculture industry.

1.4 Thesis aim and theoretical approach

The aim of the research described in this thesis is to contribute to optimizing housefly bioconversion and larval yield of manure in larval mass-rearing. The choice to use chicken manure was made in collaboration with the project's business partner Amusca, with the location of this company as well as the source of chicken manure (Rondeel chicken farm, Barneveld) being close to the experimental facilities of Wageningen University.

The information synthesized in the previous sections yields several potential approaches to optimize manure for housefly larvae. A first approach would be the supplementation of fresh manure with an easily available energy source such as starch. Starch digestion by larvae is facultative, they do not need starch and can use protein (amino acids) as energy source via gluconeogenesis. But to enhance larval growth, starch or starch containing substances could be added to manure. Increasing the overall amount of digestible energy might increase larval biomass, resulting in increased survival (more larvae) by reducing larval competition for energy and/or by bigger larvae as more protein can be used for growth. Starch supplementation could have the added benefit of potentially reducing the amount of nitrogenous waste emitted by larvae, which is greater when larvae are dependent on protein as an energy source. Larvae excrete uric acid from amino acid catabolism as nitrogenous waste, that is quickly converted to ammonia by specific bacteria in aerated and humid conditions. Formation of ammonia is unwanted as it is a waste of protein as well as an environmental burden (ammonia toxicity in rearing facilities, atmospheric nitrogen pollution). Starch can be altered by treatment with heat or sodium hydroxide that disrupts the original crystalline structure of starch, resulting in "gelatinized" starch. This material has a higher solubility and digestibility than native starch and is often used in animal feed. This makes gelatinized (corn) starch a good starting material for housefly substrate experimentation.

But positive effects of starch supplementation on larval growth might be decreased or reversed by the native microbiota of manure. The microbial community contains species that (rapidly) consume supplemented starch and decrease its availability for larvae. Microbial utilization of starch could also decrease protein availability as it could increase fixation of protein into (the wrong) microbial biomass. Microbial starch utilization could also negatively affect the (bio)chemical characteristics of the substrate in other ways, for example by lowering the pH that decreases abundance of other bacteria essential for larvae.

Thus, a second approach is minimizing competition with native fecal microbiota. This means eliminating microbiota or at least drastically reduce its abundance. This can be achieved in several ways. Using antibiotics would have potential for study purposes, as different compounds can selectively target different microbial taxa. But evidence suggests that certain antibiotics (e.g. gentamycin) decrease larval growth (Li *et al*. 2021). Also, antibiotics are highly unwanted in mass-

rearing as they could lead to residues and contribute to bacterial resistance. Gamma radiation is attractive as no denaturation of protein occurs but is very expensive, leaving heat sterilization as the most applicable method. Heat treatment of different manures is already practiced on a larger scale for the preparation of mushroom substrates (Kim *et al*. 2012, Atila 2016). However, heat sterilization could potentially decrease the nutritional value of manure for larvae by destroying essential microbiota, heat sensitive vitamins (B1, B6, B9) or enzymes.

A third approach is the timing of starch addition. If the model proposed above is correct, that young larvae feed mainly on soluble protein and older larvae mainly on bacteria that possibly contain glycogen, positive effects of starch on larval growth could be greater when supplemented later in the larval growth period. Or late supplementation could have less negative effects on young larvae, that might be more susceptible to (bio)chemical changes caused by starch supplementation at the start of the larval growth period.

A fourth approach would be the use of different larval densities. As described in the nutritional ecology section, houseflies practice mass oviposition, with larval masses enhancing substrate utilization and digestion and possibly reducing microbial composition. Housefly larval survival (eggs to harvestable larvae) was found to be low (20 to 40%) in several studies using manure substrates and is thought to be a bottleneck in development of mass-rearing (Barnard *et al*. 1998, Pastor *et al*. 2011, Cickova *et al*. 2012a). Survival of different instars in relation to nutrition has hitherto not been studied but, speculating from larval nutritional ecology, survival of early larval instars might be lower when starch is supplemented at the start of the larval growth period due to their adaptation to a protein diet, combined with the potential adverse effects of starch. But early instar survival under starch supplementation might be increased by higher larval densities, with resulting larval masses potentially increasing starch digestion and decreasing microbial competition.

1.5 Outline of the thesis

Chapter 1 of the thesis introduces the societal relevance and the opportunities and challenges of manure bioconversion by housefly larvae. Knowledge gaps are addressed and available literature on larval biology and utilization of animal manure by larvae are reviewed in order to identify potential approaches for manure manipulation. These approaches are translated into experiments described in the **Chapters 2**, **3**, **4** and **5**. **Chapter 2** concerns the starch supplementation and heat sterilization of chicken manure. The central hypothesis is that larvae will produce more biomass when fresh chicken manure is heat sterilized before starch is added. Substrates containing fructo-oligosaccharide, in theory only digestible by bacteria and not by larvae, are also investigated. Substrate and substrate residue DM, N, ammonia and uric acid content and pH are determined, together with the larval growth parameters individual larval weight, total larval mass and larval DM and N content. This allows for calculation of larval survival based on larval weights and bioconversion based on DM and N. Furthermore, the data provided insights on the generation of ammonia, the fate of uric acid and substrate pH for manure starch supplementation. **Chapter 3** concerns the dose-response relation between starch and larval growth and substrate bioconversion. The central hypothesis is that a specific starch concentration is effective to increase larval growth, with the dose dependent on native

microbial load of manure. This is tested by adding a range of starch doses to fresh and heat sterilized chicken manure, measuring the same substrate and larval parameters as in **Chapter 2**. **Chapter 4** concerns the timing of starch addition during rearing. The central hypothesis is that older larvae might be better adapted to digest starch than younger larvae. This is tested by adding an optimal starch dose (determined in **Chapter 3**) to fresh chicken manure at two different times (0 and 36 h) during the larval growth period. Glucose and cellulose were used as control. Glucose is in theory digestible by larvae of all ages, while cellulose is not or less digestible by larvae but accounts for the effects of stirring during the larval growth period. The same larval growth and substrate bioconversion parameters as in chapter 2 were measured, except for uric acid content. **Chapter 5** concerns the effect of larval density on larval growth and survival for starch supplementation at the start of the growing period. The central hypothesis is that young larvae are adversely affected by starch addition and that higher larval densities can mitigate this effect. This is tested by adding starch and glucose (control) at the start of the growth period and inoculating substrates with varying densities of eggs (8 to 20 eggs/g substrate). Samples were harvested at 2 different times (~58 and 120 h) in order to determine early and late larval growth and survival. The same larval growth and substrate bioconversion parameters were measured as in chapter 4. **Chapter 6** integrates and discusses the experimental results, ending with recommendations for the use of manure in housefly larvae mass-rearing and future studies. The **appendices** contain experimental work on housefly rearing, egg collection and larvae harvesting.

References.

Abd Manan, F., Yeoh, Y. K., Chai, T. T., Wong, F. C. (2024). Unlocking the potential of black soldier fly frass as a sustainable organic fertilizer: A review of recent studies. *Journal of Environmental Management*, 367, 121997.

Adeolu, M., Alnajar, S., Naushad, S., Gupta, R. S. (2016). Genome-based phylogeny and taxonomy of the 'Enterobacteriales': Proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae, Pectobacteriaceae, Yersiniaceae, Hafniaceae, Morganellaceae, and Budviciaceae. *International Journal of Systematic and Evolutionary Microbiology*, 66(12), 5575-5599.

Ahmad, S. (1970). Studies on aliesterase, lipase and peptidase in susceptible and organophosphate-resistant strains of house fly (*Musca domestica* L.). *Comparative Biochemistry and Physiology*, 32, 465-474.

Ajani, E. K., Nwanna, L.C., Musa, B. O. (2004). Replacement of fishmeal with maggot meal in the diets of Nile tilapia, *Oreochromis niloticus*. *World Aquaculture*, 35(1), 52-54.

Allee, W. C., Park, O., Emerson, A. E., Park, T., Schmidt, K. P. (1949). Principles of animal ecology (1st ed., pp. xii, 837). Philadelphia, PA, USA: W.B. Saunders Company.

Anees-Hill, S., Douglas, P., Pashley, C. H., Hansell, A., Marczylo, E. L. (2022). A systematic review of outdoor airborne fungal spore seasonality across Europe and the implications for health. *Science of the Total Environment*, 818, 151716.

Anene, A., Afam-Anene, C. O., Ike, K., Ekekwe, H. N. (2013). Preliminary investigations on quantity and proximate quality of maggots produced from four different sources of livestock wastes. *Journal of Research in Biology*, 3, 1060-1065.

Aniebo, A. O., Erondu, E. S., Owen, O. J. (2008). Proximate composition of housefly larvae (*Musca domestica*) meal generated from mixture of cattle blood and wheat bran. *Livestock Research for Rural Development*, 20(12), article 205.

Aarnink, A. J. A., Verstegen, M. W. A. (2007). Nutrition, key factor to reduce environmental load from pig production. *Livestock Science*, 109, 194-203.

Apajalathi, J., Vienola, K. (2016). Interaction between chicken intestinal microbiota and protein digestion. *Animal Feed Science and Technology*, 221(B), 323-330.

Atila, F. (2016). Effect of different substrate disinfection methods on the production of *Pleurotus ostreatus*. *Journal of Agricultural Studies*, 4: article 10051.

Atiyeh, R. M., Lee, S., Edwards, C. A., Arancon, N. Q., Metzger, J. D. (2002). The influence of humic acids derived from earthworm-processed organic wastes on plant growth. *Bioresource Technology*, 84(1), 7-14.

Awasthi, M. K., Liu, T., Awasthi, S. K., Duan, Y., Pandey, A., Zhang, Z. (2020). Manure pretreatments with black soldier fly *Hermetia illucens* L. (Diptera: Stratiomyidae): A study to reduce pathogen content. *Science of the Total Environment*, 737, 139842.

Baiano, A. (2020). Edible insects: An overview on nutritional characteristics, safety, farming, production technologies, regulatory framework, and socio-economic and ethical implications. *Trends in Food Science and Technology*, 100, 35-50.

Baker, F. D., Paretsky, D. (1958). Studies on the enzyme lipase in the housefly. *Archives of Biochemistry and Biophysics*, 77(2), 328-335.

van Baalen, M., Sabelis, M. W. (1995). The dynamics of multiple infection and the evolution of virulence. *The American Naturalist*, 146(6), 881-910.

Barka, E. A., Vatsa, P., Sanchez, L., Gaveau-Vaillant, N., Jacquard, C., Klenk, H.-P., Clément, C., Ouhdouch, Y., van Wezel, G. P. (2016). Taxonomy, physiology, and natural products of Actinobacteria. *Microbiology and Molecular Biology Reviews*, 80(1), 1-43.

Barnard, D. R., Geden, C.J. (1993). Influence of larval density and temperature in poultry manure on development of the house fly (Diptera: Muscidae). *Environmental Entomology*, 22, 971-977.

Barnard D. R., Harms R. H., Sloan D. R. (1998). Biodegradation of poultry manure by house fly (Diptera: Muscidae). *Environmental Entomology*, 27, 600-605.

Barnhart, C. L., Chadwick, L. E. (1953). Laboratory observations on the aggregation behavior of the house fly. *Journal of Economic Entomology*, 46(5), 900-906.

Bar-On, Y. M., Phillips, R., Milo, R. (2018). The biomass distribution on earth. *Proceedings of the National Academy of Sciences*, 115(25), 6506-6511.

Bebitha, B., Mohanraj, P., Manimegalai, S., Mahalingam, C. A. (2016). Silkworm disease diagnosis through molecular approach and their management. *International Journal of Plant Protection*, 9(1), 343-352.

van Beek, C. L., van den Eertwegh, G. A. P. H., van Schaik, F. H., Velthof, G. L., Oenema, O. (2004). The contribution of agriculture to N and P loading of surface water in grassland on peat soil. *Nutrient Cycling in Agroecosystems*, 70, 85-95.

Bender, D. A. (2014). Introduction to nutrition and metabolism (5th ed.). Boca Raton, FL, USA: CRC Press.

Berg, J. M., Tymoczko, J. L., Gatto Jr., G. J. Stryer, L. (2015). Biochemistry (8th ed.)., New York, NY, USA: W.H. Freeman & Company.

Bergmann, E. D., Levinson, Z. H. (1954). Steroid requirements of housefly larvae. *Nature*, 173(4396), 211-212.

Bessa, L. W., Pieterse, E., Marais, J., Dhanani, K., Hoffman, L. C., McDonnell, C., Buckow, R., Colgrave, M. (2021). Food safety of consuming black soldier fly (*Hermetia illucens*) larvae: Microbial, heavy metal and cross-reactive allergen risks. *Foods*, 10(8), 1934.

Black, W. C., Krafsur, E. S. (1985). Electrophoretic analysis of genetic variability in the house fly (*Musca domestica* L.). *Biochemical. Genetics*, 23(3), 193.

Blahovec, J., Kostecka, Z., Kocisova, A. (2006). Peptidolytic enzymes in different larval stadium of house fly *Musca domestica*. *Veterinarni Medicina*, 51, 139-144.

Blok, M. C., Brandsma, G., Bosch, G., Gerrits, W. J. J., Jansman, A. J. M., Fledderus, J., Everts, H. (2015). A new Dutch net energy formula for feed and feedstuffs for growing and fattening pigs (CVB-Documentation Report No. 56). Wageningen UR Livestock Research, Lelystad, The Netherlands.

Boatta, F., Jansen, W. L., Beukeboom, L. W., Ellers, J. (2023). Lipid quantity and composition vary between three European house fly strains. *Entomologia Experimentalis et Applicata*, 171(4), 248-257.

Bonelli, M., Bruno, D., Caccia, S., Sgambetterra, G., Cappellozza, S., Jucker, C., Tettamanti, G., Casartelli, M. (2019). Structural and functional characterization of *Hermetia illucens* larval midgut. *Frontiers in Physiology*, 10, 204.

Bosch, G., van Zanten, H. H. E., Zamprogna, A., Veenenbos, M., Meijer, N. P., van der Fels-Klerx, H. J., van Loon, J. J. A. (2019). Conversion of organic resources by black soldier fly larvae: Legislation, efficiency and environmental impact. *Journal of Cleaner Production*, 222, 355-363.

Broce, A. B., Haas, M. S. (1999). Relation of cattle manure age to colonization by stable sly and house fly (Diptera: Muscidae). *Journal of the Kansas Entomological Society*, 72(1), 60-72.

Brodie, B. S., Gries, R., Martins, A., Gries, G. (2015). Bacteria and fly odors: Indole as a key semiochemical mediating attraction of the blow fly *Lucilia sericata* to decaying matter. *Journal of Chemical Ecology*, 41(5), 451-460.

Brookes, F. J., Frankel G. (1958). The nutrition of the larva of the housefly. *Physiological Zoology*, 31(3), 208-223.

Cai, Z., Yang, D., Wu, S., Wang, Y., Reaney, M. J. T., Zhao, Z., Zhu, L., Sun, G., Niu, Y., Zheng, D., Niu, H., Yang, W. (2017). Conversion of poultry manure to biodiesel, a practical method of producing fatty acid methyl esters via housefly (*Musca domestica* L.) larval lipid. *Fuel*, 210, 463-471.

Callewaert, L, Michiels, C. W. (2010). Lysozymes in the animal kingdom. *Journal of Biosciences*, 35,127-160.

Canavoso, L. E., Jouni, Z. E., Karnas, K. J., Pennington, J. E., Wells, M. A. (2001). Fat metabolism in insects. *Annual Review of Nutrition*, 21(1), 23-46.

Chang, J. T., Wang, M. Y. (1958). Nutritional requirements of the common housefly, *Musca domestica vicina* Macq. *Nature*, 181, 566-568.

Charalampopoulos, D., Wang, R., Pandiella, S. S., Webb, C. (2002). Application of cereals and cereal components in functional foods: A review. *International Journal of Food Microbiology*, 79(1-2), 131-141.

Chen, S., Liao, W., Liu, C., Wen, Z., Kincaid, R. L., Harrison, J. H., Brown, M. D., Solana, A. E., Stevens, D. J. (2003). Value-added chemicals from animal manure (PNNL-14495). Pacific Northwest National Laboratory, Richland, WA, USA.

Chen, S., Wen, W., Liao, C., Liu, R., Kincaid, J., Harrison, D, Elliot, M., Brown, D., Stevens, J. (2005). Studies into using manure in a biorefinery concept. *Applied Biochemistry and Biotechnology*, 124(1-3), 999-1015.

Chinwendu, S., Chibueze, U., Tochukwu, E. (2013). Anaerobic digester considerations of animal waste. *American Journal of Biochemistry*, 3(4), 93-96.

Chu, X., Zhu, W., Li, X., Su, E., Wang, J. (2024). Bitter flavors and bitter compounds in foods: identification, perception, and reduction techniques. *Food Research International*, 183, 112234.

Čičková, H., Kozánek, M., Takáč, P. (2012a). Improvement of survival of the house fly (*Musca domestica* L.) larvae under mass-rearing conditions. *Bulletin of Entomological Research*, 103(1), 119-125.

Čičková, H., Pastor, B., Kozánek, M., Martínez-Sánchez, A., Rojo, S., Takáč, P. (2012). Biodegradation of pig manure by the housefly, *Musca domestica*: a viable ecological strategy for pig manure management. *PLoS ONE*, 7(3), e32798.

Čičková, H., Newton, G. L., Lacy, R. C., Kozánek, M. (2015). The use of fly larvae for organic waste treatment. *Waste Management*, 35, 68-80.

CLO (2024a). Ontwikkeling veestapel op landbouwbedrijven, 1980-2023 (indicator 2124, versie 13, 16 mei 2024). Centraal Bureau voor de Statistiek, Den Haag; Planbureau voor de Leefomgeving, Den Haag; Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven; Wageningen University and Research, Wageningen, The Netherlands.

CLO (2024b). Mestproductie door de veestapel, 1986-2023 (indicator 0104, versie 29, 25 maart 2024). Centraal Bureau voor de Statistiek, Den Haag; Planbureau voor de Leefomgeving, Den Haag; Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven; Wageningen University and Research, Wageningen, The Netherlands.

Cortinhas, L. B., Mendonça, P. M., Braga, M. V., de Carvalho Queiroz, M. M. (2020). Ultrastructure of the immature stages of *Musca domestica* (Diptera: Muscidae: Muscinae). *Journal of Medical Entomology*, 57(6), 1712-1721.

Curti, E., Carini, E., Bonacini, G., Tribuzio, G., Vittadini, E. (2013). Effect of the addition of bran fractions on bread properties. *Journal of Cereal Science*, 57, 325-332.

De Bianchi, A. G., Capurro, M. L., Marinotti, O. (1987). Lipophorin in the larval and adult stages of *Musca domestica*. *Archives of Insect Biochemistry and Physiology*, 6(1), 39-48.

De Lange, C. F. M., Pluske, J. Gong, J., Nyachoti, C.M. (2010). Strategic use of feed ingredients and feed additives to stimulate gut health and development in young pigs. *Livestock Science*, 134, 124-134.

De Smet, I., De Boever, P., Verstraete, W. (1998). Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. *British Journal of Nutrition*, 79(2), 185-194.

Delgado, L., Garino, C., Moreno, F. J., Zagon, J., Broll, H. (2023). Sustainable food systems: EU regulatory framework and contribution of insects to the Farm-To-Fork strategy. *Food Reviews International*, 39(9), 6955-6976.

Derrien, M., Jardé, E., Gruau, G., Pierson-Wickmann, A.C. (2011). Extreme variability of steroid profiles in cow feces and pig slurries at the regional scale: implications for the use of steroids to specify fecal pollution sources in waters. *Journal of Agricultural and Food Chemistry*, 59(13), 7294-7302.

Dethier, V. G. (1955). The physiology and histology of the blowfly's chemoreceptors. *Biological Bulletin*, 109(2), 194-204.

Devane, M. L., Wood, D., Chappell, A., Robson, B., Webster-Brown, J., Gilpin, B. J. (2015). Identifying avian sources of faecal contamination using sterol analysis. *Environmental Monitoring and Assessment*, 187(10), 1-19.

Ding, S., Li, X., Wang, N., Cameron, S. L., Mao, M., Wang, Y., Xi, Y., Yang, D. (2015). The phylogeny and evolutionary timescale of Muscoidea (Diptera: Brachycera: Calyptratae) inferred from mitochondrial genomes. *PLOS ONE*, 10(7), e0134170.

Dowding, V.M. (1967). The function and ecological significance of the pharyngeal ridges occurring in the larvae of some cyclorrhaphous Diptera. *Parasitology*, 57(2), 371-388.

Dwivedy, A.K. (1975). Dietary cholesterol requirements of housefly *Musca domestica* larvae. *Journal of Insect Physiology*, 21, 1685-1690.

Edgington, S., Thompson, E., Moore, D., Hughes, K. A., Bridge, P. (2014). Investigating the insecticidal potential of *Geomyces* (Myxotrichaceae: Helotiales) and *Mortierella* (Mortierellaceae: Mortierellales). *SpringerPlus*, 3, article 289.

El-Deek, A. A., Ghonem, K. M., Hamdy, M., Aser, M. A., Aljasass, F., Osman, M. M. (2009). Producing single cell protein from poultry manure and evaluation for broiler chickens diets. *International Journal of Poultry Science*, 8(11), 1062-1077.

Emerson, P. M., Bailey, R. L., Mahdi, O. S., Walraven, G. E., Lindsay, S. W. (2000). Transmission ecology of the fly *Musca sorbens*, a putative vector of trachoma. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 94(1), 28-32.

Eurostat (2024). Livestock density index. Accessed January 10, 2025.

FAO (2012). The state of world fisheries and aquaculture. Rome, Italy, FAO. Part 1, 3-52.

Farkas, R., Hogsette, J. A., Borzsonyi, L. (1998). Development of *Hydroatea aenescens* and *Musca domestica* (Diptera: Muscidae) in poultry and pig manures of different moisture content. *Environmental Entomology*, 27, 695-699.

Fasakin, E. A., Balogun, A. M., Ajayi, O. O. (2003). Evaluation of full fat and defatted maggot meals in the feeding of clariid catfish *Clarias gariepinus* fingerlings. *Aquaculture Research*, 34, 733-738.

Fashina-Bombata, H. A., Balogun, O. (1997). The effect of partial or total replacement of fish meal with maggot meal in the diet of tilapia (*Oreochromis niloticus*) fry. *Journal of Prospects in Science*, 1, 178-181.

Fatchurochim, S., Geden, C. J., R., Axtell, C. (1988). Filth fly (Diptera) oviposition and larval development in poultry manure of various moisture levels. *Journal of Entomological Science*, 24, 224-231.

Field, C. B., Barros, V. R., Dokken, D. J., Mach, K. J., Mastrandrea, M. D., Bilir, T. E., Chatterjee, M. (2014). Climate change, impacts, adaptation and vulnerability. Summary for policymakers. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC), 1-32.

Fu, P., Wu, J., Guo, G. (2009). Purification and molecular identification of an antifungal peptide from the hemolymph of *Musca domestica* (housefly). *Cellular and Molecular Immunology*, 6, 245-251.

Fügi, M. A., Gunasekera, K., Ochsenreiter, T., Guan, X., Wenk, M. R., Mäser, P. (2014). Genome profiling of sterol synthesis shows convergent evolution in parasites and guides chemotherapeutic attack. *Journal of Lipid Research*, 55(5), 929-938.

Gadzama, I.U., Ndudim, R.K. (2019). Nutritional composition of housefly larvae meal: a sustainable protein source for animal production - a review. *Acta Scientific Agriculture* 3, 74-77.

Ganda, H., Zannou, E. T., Kenis, M., Abihona, H. A., Houndonougbo, F. M., Chrysostome, C. A. A. M., Chougourou, D. C., Mensah, G. A. (2021). Effect of four rearing substrates on the yield and the chemical composition of housefly larvae, *Musca domestica* L. 1758 (Diptera: Muscidae). *International Journal of Tropical Insect Science*, 42(2), 1331-1339.

Geden, C. J., Nayduch, D., Scott, J. G., Burgess, E. R., Gerry, A. C., Kaufman, P. E., Thomson, J., Pickens, V., Machtinger, E. T. (2021). House fly (Diptera: Muscidae): Biology, pest status, current management prospects, and research needs. *Journal of Integrated Pest Management*, 12(1), article 39.

Godfray, H. C. J., Aveyard, P., Garnett, T., Hall, J. W., Key, T. J., Lorimer, J., Pierrehumbert, P. S., Springmann, M., Jebb, S. A. (2018). Meat consumption, health, and the environment. *Science*, 361, 6399-7407.

Greenberg, B. (1971). Flies and disease (Vol. I). Princeton, NJ, USA: Princeton University Press.

Greenberg, B., Paretsky, D. (1955). Proteolytic enzymes in the house fly, *Musca Domestica* (L.). *Annals of the Entomological Society of America*, 48, 46-50.

Greenspoon, L., Krieger, E., Sender, R., Rosenberg, Y., Bar-On, Y. M., Moran, U., Antman, T., Meiri, S., Roll, U., Noor, E., Milo, R. (2023). The global biomass of wild mammals. *Proceedings of the National Academy of Sciences of the United States of America*, 120(10), e2204892120.

Hashizume, A., Ido, A., Ohta, T., Thiaw, S. T., Morita, R., Nishikawa, M., Takahashi, T., Miura, C., Miura, T. (2019). Housefly (*Musca domestica*) larvae preparations after removing the hydrophobic fraction are effective alternatives to fish meal in aquaculture feed for red seabream (*Pagrus major*). *Fishes*, 4(3), article 38.

Haseyama, K. L. F., Wiegmann, B. M., Almeida, E. A. B., de Carvalho, C. J. B. (2015). Say goodbye to tribes in the new house fly classification: A new molecular phylogenetic analysis and an updated biogeographical narrative for the Muscidae (Diptera). *Molecular Phylogenetics and Evolution*, 89, 1-12

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H. (2015). "Rumen microbial community composition varies with diet and host." *Scientific Reports,* 5, 14567.

Hewitt, C.G. (1910). The house fly. A study of its structure, development, bionomics and economy, pp. 395-448. Manchester, UK: University Press.

Hilliard, E. P., Beard, J., Pearce, G. R. (1979). Utilization of piggery waste I. The chemical composition and *in vitro* organic matter digestibility of pig faeces from commercial piggeries in south-eastern Australia. *Agriculture and Environment*, 4(3), 171-180.

Hirashima, A., Oyama, K., Ueno, R., Ishaaya, I., Eto, M. (1989). Carbohydrate enzymes in house fly *Musca domestica* larvae: biochemical properties and toxicological significance. *Journal of the Faculty of Agriculture*, Kyushu University, 34(1/2), 37-42.

Hogsette, J.A. (1992). New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. *Journal of Economical Entomology*, 85(6), 2291-2294.

Holl, P., Gries, G. (2018). Semiochemicals of house flies (*Musca domestica* L.) mediate attraction and aggregation. *Frontiers in Ecology and Evolution*, 6, 196.

Hu, R., Zhang, S., Wu, J. W., Guo, G., Ping, F. (2013). Detection of cellulases and the relationship between enzyme activity and development of *Musca domestica*. *Chinese Journal of Applied Biology*, 31(4), 256-61 (English abstract).

Hussein, M., Pillai, V. V., Goddard, J. M., Park, H. G., Kothapalli, K. S., Ross, D. A., Selvaraj, V. (2017). Sustainable production of housefly (*Musca domestica*) larvae as a protein-rich feed ingredient by utilizing cattle manure. PLoS ONE, 12(2), e0171708

Hwangbo, J., Hong, E. C., Jang, A., Kang, H. K., Oh, J. S., Kim, B. W., Park, B. (2008). Utilization of house fly-maggots, a feed supplement in the production of broiler chickens. *Journal of Environmental Biology*, 30, 609-614.

Intayung, D., Chundang, P., Srikachar, S., Kovitvadhi, A. (2021). Ontogenic development of the digestive enzymes and chemical composition of *Hermetia illucens* larvae of different ages. *Entomologia Experimentalis et Applicata*, 169(7), 665-673.

Ito, Y., Nakamura, M., Hotani, T., Imoto, T. (1995). Insect lysozyme from house fly (*Musca domestica*) larvae: possible digestive function based on sequence and enzymatic properties. *Journal of Biochemistry*, 118, 546-551.

Jiang, Y., Lei, C., Niu, C., Fang, Y., Xiao, C., Zhang, Z. (2002). Semiochemicals from ovaries of gravid females attract ovipositing female houseflies, *Musca domestica*. *Journal of Insect Physiology*, 48(10), 945-950.

Jing, X., Behmer, S. T. (2020). Insect sterol nutrition: physiological mechanisms, ecology and applications. *Annual Review of Entomology*, 65, 251-271.

Johnson, A. P., Wallman, J. F. (2014). Effect of massing on larval growth rate. *Forensic Science International*, 241, 141-149.

Jordão, B. P, Terra, W. R, Ribeiro, A. F., Lehane, M. J., Ferreira, C. (1996). Trypsin secretion in *Musca domestica* larval midguts: A biochemical and immunocytochemical study. *Insect Biochemistry and Molecular Biology*, 26(4), 337-346.

Junqueira, A. C. M., Ratan, A., Acerbi, E., Drautz-Moses, D. I., Premkrishnan, B. N. V., Costea, P. I., Chen, X., Tettelin, H., Segata, N., Schuster, S. C. (2017). The microbiomes of blowflies and houseflies as bacterial vectors. *Scientific Reports*, 7, 16324.

Kakuru, J. B., Semwanga, N., Kasigwa, H., Kirunda, H., Kemigabo, C. (2022). Effect of substrate and rearing period on the yield and quality of housefly larvae (*Musca domestica*) as protein ingredient in fish diets. *International Journal of Innovative Science and Research Technology*, 7(5), 826-829.

Kanost, M. R., Clem, R. J. (2012). Insect proteases. In L. I. Gilbert (Ed.), Insect molecular biology and biochemistry (pp. 346-364). Academic Press.

Kaplanis, J. N., Robbins, W. E., Tabor, L. A. (1960). The utilization and metabolism of 4-C14-cholesterol by the adult house fly. *Annals of the Entomological Society of America*, 53(2), 260-264.

Khan, S., Khan, R.U., Sultan, A., Khan, M., Hayat, S.U., Shahid, M.S. (2016). Evaluating the suitability of maggot meal as a partial substitute of soya bean on the productive traits, digestibility indices and organoleptic properties of broiler meat. *Journal of Animal Physiology and Animal Nutrition*, 100, 649-656.

Kim, J., Diao, J., Shepherd, M. W. Jr., Singh, R., Heringa, S. D., Gong, C., Jiang, X. (2012). Validating thermal inactivation of *Salmonella* spp. in fresh and aged chicken litter. *Applied and Environmental Microbiology*, 78(4), 1302-1307.

King, J. (2019). Medical and veterinary entomology (G. R. Mullen & L. A. Durden, Eds.; Third edition). Cambridge, MA, USA: Academic Press.

Klug, C., Fehlhaber, K., Müller, U., Braun, P. (1998). Kombinierter Einfluss von Temperatur und pH-Wert auf die Aktivität der Proteasen von *Pseudomonas*- und *Bacillus*-Stämmen. *Berliner und Münchener tierärztliche Wochenschrift*, 111(1), 9-12.

Kobayashi, M., Sasaki, T., Saito, N., Tamura, K., Suzuki, K., Watanabe, H., Agui, N. (1999). Houseflies: Not simple mechanical vectors of enterohemorrhagic *Escherichia coli* O157:H7. *American Journal of Tropical Medicine and Hygiene*, 61(4), 625-629.

Koerkamp, P. W. G. (1994). Review on ammonia emission from housing systems for laying hens in relation to sources, processes, building design and manure handling. *Journal of Agricultural Engineering Research*, 59, 73-87.

Kutty, S. N., Pape, T., Pont, A., Wiegmann, B. M., Meier, R. (2008). The Muscoidea (Diptera: Calyptratae) are paraphyletic: Evidence from four mitochondrial and four nuclear genes. *Molecular Phylogenetics and Evolution*, 49(2), 639-652.

Lam, K., Geisreiter, C., Gries, G. (2009). Ovipositing female house flies provision offspring larvae with bacterial food. *Entomologia Experimentalis et Applicata*, 133, 292-295.

Lam, K., Thu, K., Tsang, M., Moore, M., Gries, G. (2009a) Bacteria on house fly eggs, *Musca domestica*, suppress fungal growth in chicken manure through nutrient depletion or antifungal metabolites. *Naturwissenschaften*, 96, 1127-1132.

Larraín, S. P., Salas, F. C. (2008). House fly (*Musca domestica* L.) (Diptera: Muscidae) development in different types of manure. *Chilean Journal of Agricultural Research*, 68, 192-197.

Lawrence, P. K., Koundal, K. R. (2002). Plant protease inhibitors in control of phytophagous insects. *Electronic Journal of Biotechnology*, 5(1), 5-6.

Lee, J., Kim, Y. K., Kim, D., Suh, S. J. (2023). Taxonomy of the family Xylomyidae (Diptera: Stratiomyoidea) from Korea. *Journal of Asia-Pacific Biodiversity*, 16(1), 43-48.

Leeming, R., Ball, A., Ashbolt, N., Nichols, P. (1996). Using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters. *Water Research*, 30(12), 2893-2900.

Leeson, S., Summers, J. D. (2008). Commercial Poultry Nutrition (3rd ed.). Guelph, ON, Canada: University Books.

Lemos, F. J., Terra, W. R. (1991). Digestion of bacteria and the role of midgut lysozyme in some insect larvae. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology*, 100(2), 265-268.

Lewin, G. R., Carlos, C., Chevrette, M. G., Horn, H. A., McDonald, B. R., Stankey, R. J., Fox, B. G., Currie, C. R. (2016). Evolution and ecology of Actinobacteria and their bioenergy applications. *Annual Review of Microbiology*, 70, 235-254.

Li, T., Zhang, Q., Zhang, X., Wan, Q., Wang, S., Zhang, R., Zhang, Z. (2021). Transcriptome and microbiome analyses of the mechanisms underlying antibiotic-mediated inhibition of larval development of the saprophagous insect *Musca domestica* (Diptera: Muscidae). *Ecotoxicology and Environmental Safety*, 223, article 112602.

Li, X., Rahimnejad, S., Wang, L., Lu, K., Song, K., Zhang, C. (2019). Substituting fish meal with housefly (*Musca domestica*) maggot meal in diets for bullfrog *Rana* (*Lithobates*) *catesbeiana*: Effects on growth, digestive enzymes activity, antioxidant capacity and gut health. *Aquaculture*, 499, 295-305.

Liu, P. V., Hsieh, H. C. (1969). Inhibition of protease production of various bacteria by ammonium salts: its effect on toxin production and virulence. *Journal of Bacteriology*, 99(2), 406-413.

Liu, Y., Chen, Y., Wang, N., Qin, H., Zhang, L., Zhang, S. (2023). The global prevalence of parasites in non-biting flies as vectors: a systematic review and meta-analysis. *Parasites & Vectors*, 16(1), 25.

Loughrin, J. H., Szogi, A. A. (2006). Free fatty acids and sterols in swine manure. *Journal of Environmental Science and Health Part B*, 41(1), 31-42.

Lussenhop, J., Kumar, R., Wicklow, D.T., Lloyd, J.L. (1980). Insect effects on bacteria and fungi in cattle dung. *Oikos*, 34, 54-58.

Lysyk, T. J., Kalischuk-Tymensen, L., Selinger, L. B., Lancaster, R .C., Wever, L., Cheng, K. J. (1999). Rearing stable fly larvae (Diptera: Muscidae) on an egg-yolk medium. *Journal of Medical Entomology*, 36, 382-388.

MacDonald, G. K., Bennett, E. M., Potter, P. A., Ramankutty, N. (2011). Agronomic phosphorus imbalances across the world's croplands. *Proceedings of the National Academy of Sciences of the United States of America*, 108(7), 3086-3091.

Maczkowiak, F., Lage, J.-L. (2006). Origin and evolution of the Amyrel gene in the α-amylase multigene family of Diptera. *Genetica*, 128(1-3), 145-158.

Makkar, H. P. S., Tran, G., Heuzé, V., Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology*, 197, 1-33.

Martin, J. H., Loehr, R. C., Pilbeam, T. E. (1983). Animal manures as feedstuffs: Nutrient characteristics. *Agricultural Wastes*, 6, 131-166.

Maślanko, W., Szwaj, E., Gazda, M., Bartosik, K. (2022). *Hippobosca equina* L. (Hippoboscidae), an old enemy as an emerging threat in the Palearctic zone. *International Journal of Environmental Research and Public Health*, 19(24), 16978.

McCommas, S., Shornick, L. (1990). The effect of carbohydrate sources on the level of amylase activity in *Musca domestica*. *Biochemical Genetics*, 28(11/12), 585-589.

McIntyre, G. S., Gooding, R. H. (2000). Egg size, contents and quality: maternal age and size effects on house fly eggs. *Canadian Journal of Zoology*, 78 (9), 1544-1551.

Miller, B. F., Teotia, J. S., Thatcher, T. O. (1974). Digestion of poultry manure by *Musca domestica*. *British Poultry Science* 15(2), 231-234.

Minde, D. P., Maurice, M. M., Rüdiger, S. G. (2012). Determining biophysical protein stability in lysates by a fast proteolysis assay. *PLoS ONE*, 7(10), e46147.

Miranda, C. D., Cammack, J. A., Tomberlin, J. K. (2023). Large-scale production of house fly, *Musca domestica* (Diptera: Muscidae), larvae fed 3 manure types. *Journal of Economic Entomology*, 116(4), 1102-1109.

Miyamoto, T., Amrein, H. (2017). Gluconeogenesis: An ancient biochemical pathway with a new twist. *Fly*, 11(3), 218-223.

Møller, H. B, Moset, V., Brask, M., Weisbjerg, M. R, Lund, P. (2014). Faeces composition and manure derived methane yield from dairy cows: influence of diet with focus on fat supplement and roughage type. *Atmospheric Environment*, 94, 36-43.

Mönch-Tegeder, M., Lemmer, A., Oechsner, H. (2014). Enhancement of methane production with horse manure supplement and pretreatment in a full-scale biogas process. *Energy*, 73, 523-530.

Monroe, R. E. (1959). Role of cholesterol in house fly reproduction. *Nature*, 184, 1513-1516.

Moon, R.D, Hinton, J.L., O'Rourke, S.D., Schmidt, D.R. (2001). Nutritional value of fresh and composted poultry manure for house fly (Diptera: Muscidae) larvae. *Journal of Economical Entomology*, 94(5), 1308-1317.

Moreira, C. K.,. Capurro, M. L, Calvo, E., Silva, P. I.., James, A. A., de Bianchi, A. G., Marinotti O. (2003). The *Musca domestica* larval hexamerin is composed of multiple, similar polypeptides. *Insect Biochemistry and Molecular Biology*, 33, 389-395.

Mottet, A., de Haan, C., Falcucci, A., Tempio, G., Opio, C., Gerber, P. (2017). Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. *Global Food Security*, 14, 1-8.

Muszewska, A., Okrasińska, A., Steczkiewicz, K., Drgas, O., Orłowska, M., Perlińska-Lenart, U., Aleksandrzak-Piekarczyk, T., Szatraj, K., Zielenkiewicz, U., Piłsyk, S., Malc, E., Mieczkowski, P., Kruszewska, J. S., Bernat, P., Pawłowska, J. (2021). Metabolic potential, ecology and presence of associated bacteria is reflected in genomic diversity of Mucoromycotina. *Frontiers in Microbiology*, 12, article 636986.

Nayduch, D., Burrus, R. G. (2017). Flourishing in filth: house fly-microbe interactions across life history. *Annals of the Entomological Society of America*, 110(1), 6-18.

Nayduch, D., Joyner, C. (2013). Expression of lysozyme in the life history of the house fly (*Musca domestica* L.). *Journal of Medical Entomology*, 50(4), 847-852.

Niode, N. J., Mahono, C. K., Lolong, F. M., Matheos, M. P., Kepel, B. J., Tallei, T. E. (2022). A review of the antimicrobial potential of Musca domestica as a natural approach with promising prospects to countermeasure antibiotic resistance. *Veterinary Medicine International*, 2022, 9346791.

Nodar, R., Acea, M. J., Carballas, T. (1990). Microbial composition of poultry excreta. *Biological Wastes*, 33(2), 95-105.

O'Hara, C. M., Brenner, F. W., Miller, J. M. (2000). Classification, identification and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clinical Microbiology Reviews, 13(4), 534-546.

Oladele, P., Ngo, J., Chang, T., Johnson, T. A. (2024). Temporal dynamics of fecal microbiota community succession in broiler chickens, calves, and piglets under aerobic exposure. *Microbiology Spectrum*, 12(6), e0408423.

Omalu I., Ayanwale V., Ajalaruru A., Mohammed A., Bala J., Chukwuemeka V. (2009). Isolation of fungi and bacteria from housefly (*Musca Domestica* L.) larvae. *The Internet Journal of Microbiology*, 2009, 9, article 1.

Oonincx, D. G. A. B. (2015). Insects as food and feed: nutrient composition and environmental impact. PhD thesis Wageningen University, Wageningen, The Netherlands.

Pastor, B., Čičková, H., Kozánek, M., Martínez-Sánchez, A., Takáč, P., Rojo, S. (2011) Effect of the size of the pupae, adult diet, oviposition substrate and adult population density on egg production in *Musca domestica* (Diptera: Muscidae). *European Journal of Entomology* 108, 587-596.

Pava-Ripoll, M., Pearson, R. E. G., Miller, A. K., Tall, B. D., Keys, C. E., Ziobro, G. C. (2015). Ingested *Salmonella enterica*, *Cronobacter sakazakii*, *Escherichia coli* O157: H7 and *Listeria monocytogenes*: transmission dynamics from adult house flies to their eggs and first filial (F1) generation adults. *Microbiology*, 15, 1-12.

Pearson, M. M., Yep, A., Smith, S. N., Mobley, H. L. T. (2011). Transcriptome of *Proteus mirabilis* in the murine urinary tract: virulence and nitrogen assimilation gene expression. *Infection and Immunity*, 79, 2619-2631.

Perri, D. V., Bruzzone, O., Easdale, M. H. (2023). Ecological relationships between coprophagous insects and livestock production: a review. *Bulletin of Entomological Research*, 113(6), 735-747.

Perry, A. S., Miller, S. (1965). The essential role of folic acid and the effect of antimetabolites on growth and metamorphosis of housefly larvae *Musca domestica* L. *Journal of Insect Physiology*, 11, 1277-1287.

Pieterse, E., Pretorius, Q., Hoffman, L. C., Drew, D. W. (2014). The carcass quality, meat quality and sensory characteristics of broilers raised on diets containing either *Musca domestica* larvae meal, fish meal or soya bean meal as the main protein source. *Animal Production Science*, 54, 622-628.

Pimentel, A. C., Barroso, I. C., Ferreira, J. M. J., Dias, R. O., Ferreira, C., Terra, W. R. (2018). Molecular machinery of starch digestion and glucose absorption along the midgut of *Musca domestica*. *Journal of Insect Physiology*, 109, 11-20.

Prager, E. M., Jollès, P. (1996). Animal lysozymes c and g: An overview. In P. Jollès (Ed.), Lysozymes: Model enzymes in biochemistry and molecular biology. Basel, Switzerland: Birkhäuser Verlag.

Pretorius, Q. (2011). The evaluation of larvae of *Musca domestica* (common house fly) as protein source for broiler production. Thesis M.Sc. Faculty of Agricultural Sciences, University of Stellenbosch, Stellenbosch, South Afrika.

Rauch, R., Martín-Tereso, J., Daniel, J. B., Dijkstra, J. (2021). Dietary protein oscillation: Effects on feed intake, lactation performance, and milk nitrogen efficiency in lactating dairy cows. *Journal of Dairy Science*, 104(10), 10714-10726.

Richardson, H. H. (1932). An efficient medium for rearing houseflies throughout the year. *Science*, 76, 350-351.

Ritchie, H., Rosado, P., Roser, M. (2022). "Environmental impacts of food production" Published online at OurWorldinData.org. Retrieved from: 'https://ourworldindata.org/environmental-impacts-of-food' [Accessed June 12, 2023]

Rivers, D. B., Thompson, C., Brogan, R. (2011). Physiological trade-offs of forming maggot masses by necrophagous flies on vertebrate carrion. *Bulletin of Entomological Research*, 101, 599-611.

Rochon, K., Lysyk, T. J., Selinger, L. B. (2004). Persistence of *Escherichia coli* in immature house fly and stable fly (Diptera: Muscidae) in relation to larval growth and survival. *Journal of Medical Entomology*, 41, 1082-1089.

Rochon, K., Lysyk, T. J., Selinger, L .B. (2005). Retention of *Escherichia coli* by house fly and stable fly (Diptera: Muscidae) during pupal metamorphosis and eclosion. *Journal of Medical Entomology* 42, 397-403.

Rosenkranz, P., Aumeier, P., Ziegelmann, B. (2010). Biology and control of *Varroa destructor*. *Journal of Invertebrate Pathology*, 103, 96-119.

Russell, J. B., Rychlik, J. L. (2001). "Factors that alter rumen microbial ecology." *Science*, 292(5519), 1119-1122.

Sanchez-Muros M., Barroso F.G., Manzano-Agugliaro F. (2014). Insect meal as renewable source of food for animal feeding: a review. *Journal of Cleaner Production*, 65, 16-27.

Schmidtmann, E. T., Martin, P. A. W.(1992). Relationship between selected bacteria and the growth of immature house flies, *Musca domestica*, in an axenic test system. *Journal of Medical Entomology*, 29(2), 232-235.

Schulze, J., Sonnenborn, U. (2009). Yeasts in the gut: from commensals to infectious agents. *Deutsches Ärzteblatt International*, 106(51-52), 837-842.

Schuster, G. L., Donaldson, J. R., Buntyn, J. O., Duoss, H. A., Callaway, T. R., Carroll, J. A., Falkenberg, S. M., Schmidt, T. B. (2013). Use of bioluminescent *Escherichia coli* to determine retention during the life cycle of the housefly, *Musca domestica* (Diptera: Muscidae, L). *Foodborne Pathogens and Disease*, 10(5), 442-447.

Schwab, G. C., Broderick, G. A. (2017). A 100-Year Review: Protein and amino acid nutrition in dairy cows. *Journal of Dairy Science*, 100, 10094-10112.

Scott, J. G., Warren, W. C., Beukeboom, L. W., Bopp, D., Clark, A. G., Giers, S. D., Hediger, M., Jones, A. K., Kasai, S., Leichter, C. A., Li, M., Meisel, R. P., Minx, P., Murphy, T. D., Nelson, D. R., Reid, W. R., Rinkevich, F. D., Robertson, H. M., Sackton, T. B., Sattelle, D. B., Thibaud-Nissen, F., Tomlinson, C., van de Zande, L., Walden, K. K. O., Wilson, R. K., Liu, N. (2014). Genome of the house fly, *Musca domestica* L., a global vector of diseases with adaptations to a septic environment. *Genome Biology* 15, 466.

Seravalli, J., Portugal, F. (2023). Putrescine detected in strains of *Staphylococcus aureus*. *Pathogens*, 12(7), 881.

Serra, H., Costa, M. I. S., Godoy, W. A. C. (2011). Allee effect in exotic and introduced blowflies. *Neotropical Entomology*, 40(5), 519-528.

Serra, V., Pastorelli, G., Tedesco, D. E. A., Turin, L., Guerrini, A. (2024). Alternative protein sources in aquafeed: Current scenario and future perspectives. *Veterinary and Animal Science*, 25.

Silverman, P. H., Levinson, Z. H. (1954). Lipid requirements of the larvae of the housefly *Musca vicina* reared under non-aseptic conditions. *Biochemical Journal*, 58(2), 291-294.

Schmidtmann, E. T., Martin, P. A. W.(1992). Relationship between selected bacteria and the growth of immature house flies, *Musca domestica*, in an Axenic Test System. *Journal of Medical Entomology*, 29(2), 232-235.

Skidmore, P. (1985). The biology of the Muscidae of the world. Dordrecht, Holland: Junk publishers.

Spiller, D. (1964). Nutrition and diet of muscoid flies. *Bulletin of the World Health Organization*, 31, 551.

Stefanov, K., Nechev, J., Lavchieva-Nacheva, G., Nikolova, N., Seizova, K., Kwartirnikov, M., Lavchiev, V., Popov, S. (2002). Lipids and sterols in *Musca domestica* L. (Diptera, Muscidae): changes after treatment with sucrose and lead. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology*, 131(3), 543-550.

Sturgill, G., Rather, P. N. (2004). Evidence that putrescine acts as an extracellular signal required for swarming in *Proteus mirabilis*. *Molecular Microbiology*, 51(2), 437-446.

Svihus, B. (2014). Starch digestion capacity of poultry. *Poultry Science*, 93(9), 2394-2399.

Szpila, K., Pape, T. (2008). Morphology of the first instar of the house fly *Musca domestica* (Diptera: Muscidae). *Journal of Medical Entomology*, 45(4), 594-599.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., de Haan, C. D. (2006). Livestock's long shadow: environmental issues and options. Rome, Italy: Food and Agriculture Organization of the United Nations.

Terra, W. R., Espinoza-Fuentes, F. P., Ferreira, C. (1988). Midgut amylase, lysozyme, aminopeptidase, and trehalase from larvae and adults of *Musca domestica*. *Archives of Insect Biochemistry and Physiology*, 9(4), 283-297.

Terra, W., Fereirra, C. (1994). Insect digestive enzymes: properties, compartmentalization and function. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology*, 109(1), 1-62.

Thotagamuwa, A., Noriega, J. A., Webb, S., Weston, P., Doube, B. M., Caron, V., Gleeson, P., Forgie, S. A., Favila, M. E., Tomberlin, J. K., Sheppard, D. C., Joyce, J. A. (2002). Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. *Annals of the Entomological Society of America*, 95(3), 379-386.

Ulyshen, M. D. (2016). Wood decomposition as influenced by invertebrates. *Biological Reviews of the Cambridge Philosophical Society*, 91(1), 70-85.

Uriel, Y., Gries, R., Tu, L., Carroll, C,, Zhai, H., Moore, M., Gries, G. (2020) The fly factor phenomenon is mediated by interkingdom signalling between bacterial symbionts and their blow fly hosts. *Insect Science*, 27(2), 256-265.

United Nations, Department of Economic and Social Affairs, Population Division. (2024). *World Population Prospects 2024: Summary of Results*. United Nations.

van Eenennaam, A. L. (2024). Addressing the 2050 demand for terrestrial animal source food. *Proceedings of the National Academy of Sciences of the United States of America*, 121(13), e2319001121.

van Selm, B., Hijbeek, R., van Ittersum, M. K., van Hal, O., van Middelaar, C. E., de Boer, I. J. M. (2023). Recoupling livestock and feed production in the Netherlands to reduce environmental impacts. *Science of the Total Environment*, 899, 165540.

van Vliet, P. C. J., Reijs, J. W., Bloem, J., Dijkstra, J., de Goede, R. G. M. (2007). Effects of cow diet on the microbial community and organic matter and nitrogen content of feces. *Journal of Dairy Science*, 90, 5146-5158.

Wang, J. M., Liu, Q., Hou, Y., Qin, W., Bai, Z. H., Zhang, F. S., Oenema, O. (2022). Impacts of international food and feed trade on nitrogen balances and nitrogen use efficiencies of food systems. *Science of the Total Environment*, 838.

Wang, S., Huang, Z., Wan, Q., Feng, S., Xie, X., Zhang, R., Zhang, Z. (2020). Comparative genomic and metabolomic analyses of two Pseudomonas aeruginosa strains with different antifungal activities. *Frontiers in Microbiology*, 11, 1841.

Wang, H., Zhang, Z., Czapar, G., Winkler, M. K., Zheng, J. (2013). A full-scale house fly (Diptera: Muscidae) larvae bioconversion system for value-added swine manure reduction. *Waste Management and Research* 31, 223-231.

Wang, B., Wei, P. W., Yao, Y., Song, C. R., Wang, X., Yang, Y. X., Liu, H. M. (2022). Functional and expression characteristics identification of Phormicins, novel AMPs from *Musca domestica* with anti-MRSA biofilm activity, in response to different stimuli. *International Journal of Biological Macromolecules*, 209, 299-314.

World Economic Forum. (2023). The protein transformation: A roadmap for the future of alternative proteins. Geneva, Switzerland: World Economic Forum.

Wiegmann, B. M., Tsaur, S.-C., Webb, D. W., Yeates, D. K., Cassel, B. K. (2000). Monophyly and relationships of the Tabanomorpha (Diptera: Brachycera) based on 28S ribosomal gene sequences. *Annals of the Entomological Society of America*, 93(5), 1031-1038.

Woodley, N. E. (2001). A world catalog of the Stratiomyidae: (Insecta: Diptera). North American Dipterists' Society. Leiden, The Netherlands: Backhuys Publishers.

Xie, G., Kong, X., Kang, J., Su, N., Fei, J., Luo, G. (2021). Fungal community succession contributes to product maturity during the co-composting of chicken manure and crop residues. *Bioresource Technology*, 328, 443.

Yang, Z., Muhayodin, F., Larsen, O. C., Miao, H., Xue, B., Rotter, V. S. (2021). A review of composting process models of organic solid waste with a focus on the fates of C, N, P and K. *Processes,* 9(3), 473.

Yeates, D. K., Wiegmann, B. M., Courtney, G. W., Meier, R., Lambkin, C., Pape, T. (2007). Phylogeny and systematics of Diptera: Two decades of progress and prospects. *Zootaxa*, 1668(1), 565-590.

Zainudin, M. H. M., Singam, J. T., Sazili, A. Q. A. Q., Mutalib, S. A., Hassan, L. (2022). Indigenous cellulolytic aerobic and facultative anaerobic bacterial community enhanced the composting of rice straw and chicken manure with biochar addition. *Scientific Reports* 12, 5930.

Zhang, D., Sun, J., Wang, D., Peng, S., Wang, Y., Lin, X., Yang, X., Hua, Q., Wu, P. (2023). Comparison of bacterial and fungal communities structure and dynamics during chicken manure and pig manure composting. *Environmental Science and Pollution Research International*, 30, 94347-94360.

Zheng, W., Dong, Z. G., Wang, X. Q., Cao, M., Yan, B. L., Li, S. H. (2010). Effects of dietary fly maggot Musca domestica on growth and body compositions in Chinese shrimp *Fenneropenaeus chinensis* juveniles. *Fishery Science*, 29, 187-192.

Zhou, G., Wang, J., Zhu, X., Wu, Y., Gao, M., Shen, H. (2014). Induction of maggot antimicrobial peptides and treatment effect *in Salmonella pullorum*- infected chickens, *Journal of Applied Poultry Research*, 23(3), 376-383.

Zuidhof, M. J., Molnar, C. L., Morley, F. M., Wray, T. L., Robinson, F. E., Khan, B. A., Al-Ani, L., Goonewardene, L. A., (2003). Nutritive value of house fly (*Musca domestica*) larvae as a feed supplement for turkey poults. *Animal Feed Science and Technology*, 105, 225-230

Chapter 2

Bioconversion of fresh chicken excreta by housefly larvae (*Musca domestica* L.) in relation to excreta sterilization and carbohydrate addition

L.W. Pisa, D.G.A.B. Oonincx, G. Bosch, W.H. Hendriks

Journal of Insects as Food and Feed (2022), 8(10), 1107-1116

Abstract

Housefly larvae can be reared on manure and used as animal feed. Larvae can utilize proteins and easily digestible carbohydrates and have a largely unknown relation with microorganisms. The hypothesis addressed in this study was that larvae compete with microorganisms for easily digestible carbohydrates such as starch. This was tested by adding starch or (non-digestible) fructo-oligosaccharides (FOS) to unsterilised or heat-sterilised fresh chicken excreta. Experimental substrates were unsterilised excreta (E), sterilised excreta (sE) with either starch (E + star, sE + star) or FOS (E + FOS, sE + FOS). The highest wet yield (9.7 g) and heaviest larvae (13.2 mg) were on sE + star, followed by E (7.2 g and 8.1 mg). Both E + FOS and sE + FOS had minimal yields and larval weights (0.3 and 0.2 g, 2.9 and 1.7 mg) with E + star intermediate (3.3 g and 7.5 mg). Survival differed between diets, sE and E averaged 70%, sE + star 57%, E + star 33% with 9% for E + FOS and sE + FOS. DM bioconversion decreased from E (3.5%), sE + star (3.1%), sE (2.6%), E + star (1%) to E + FOS and sE + FOS (0.1%). Nitrogen bioconversion was highest on sE + star (9.9%) and E (9.3%), lower on sE (6.6%), E + star (4%) and the lowest on E + FOS and sE + FOS (0.5%). Different substrate temperature profiles during the larval growth period and different larval size distributions were found for the different substrates. The results support the hypothesis that larvae compete with microorganisms for an easily digestible carbohydrate (starch).

Introduction

Population growth and rising per-capita income are predicted to lead to increased global consumer demand for animal-derived protein by 50 to 70% over the period 2005-2050 (Alexandratos and Bruinsma 2012, Henchion *et al*. 2017). This will drive both the demand for livestock feed and emission and pollution problems associated with increased production of livestock manure (Nicholson *et al*. 2005, Godfray *et al*. 2018). Production of poultry has expanded fivefold over the last half century, with 2018 production estimated at 114 million tons of meat and 76 million tons of eggs by 23 billion animals (FAOSTAT 2020, Statista 2020). Global excretion of nitrogen (N) from poultry manure in 2017 has been estimated at 5.65 million tons for broilers and 2.36 million tons for layers (FAOSTAT 2020).

Mass rearing of insects on manure could provide protein for livestock feed and a partial solution to the environmental burden of the growing manure surplus . Housefly larvae (*Musca domestica* L.) have been reared successfully on chicken, pig and cattle manure at a laboratory and industrial scale (Calvert *et al*. 1970, Miller *et al*. 1974, Barnard *et al*. 1998, Cickova *et al*. 2012, Zhang *et al*., 2014, Hussein *et al*. 2017). These larvae are rich in crude protein (30 - 60% of the dry matter, DM) and fat (14-30% DM), with an amino acid profile comparable to fish meal (e.g. Pieterse and Pretorius 2014, Gadzama and Ndudim 2019). Larvae and defatted larvae meal can be used as an alternative for fish meal in diets of poultry, pigs and fish (Ogunji *et al*. 2008, Pretorius 2011, Veldkamp *et al*. 2012, Hashizume *et al*. 2019).

Despite the suitability and bioconversion potential of chicken manure, surprisingly little is known about its nutritional value for larvae and how manure should be treated to optimize yield and bioconversion. Fresh chicken excreta is a mixture of undigested dietary remains, endogenous material (gut and urinary mucous and secretions) and microbial mass. Fresh chicken manure commonly has a C/N of approximately 8/1, a total N content varying from 3 to 8% of DM with up to 60% of the N present as uric acid (Wang *et al*. 2015, Nahm 2003, Chen *et al*. 2005). Digestible carbohydrate content is low and consists of dietary remains especially fiber and bacterial glycogen (Weurding *et al*. 2001, Wilson *et al*. 2010). Recalcitrant plant fiber content (hemicellulose, cellulose and lignin) has been found to constitute up to 35% of excreta dry mass (Chen *et al*. 2005, Rehman *et al*. 2017).

Microbial mass of chicken excreta has not been quantified but bacterial densities of 107 to 1011 microorganisms per g excreta have been reported (Nodar *et al*. 1990, Apajalahti *et al*. 2004). Typically, bacteria (i.e. *Bacillus* and *Escherichia* sp.) found in the chicken colon contain protein (50-80% DM), ribonucleic acids from RNA or DNA (10-20% DM), a limited amount of fat (< 9% DM) and glycogen (< 5% DM) (Kurbanoglu and Algur 2002, Metcalf *et al*. 2004, Wilson *et al*. 2010, Ritala *et al*. 2017, Sekar *et al*. 2020). When excreta leave the body, a die-off of obligate anaerobic microorganisms takes place, releasing microbial cell content into the fecal mass (Ben-Amor *et al*. 2005). Fresh excreta exposed to oxygen at room temperature are rapidly colonized by different microorganisms. These microorganisms rapidly utilize easily digestible carbohydrates and protein (Steger *et al*. 2005) potentially resulting in a competition between housefly larvae and microorganisms for nutrients.

Microbial protein, along with the undigested dietary and endogenous protein, is a potential source of amino acids for larvae. The protein (and uric acid) in excreta can also be used for novel microbial growth and energy, with excess N emitted as ammonia (Maeda *et al*. 2011). Ammonia (NH_3) production due to microbial hydrolysis of proteins and breakdown of uric acid in chicken manure can be substantial, leading to high volatilization of N (Nahm 2003). Microbial use of N highly depends on the C/N, moisture content, temperature and aeration (Kutzner 2008). Generally, a low C/N (below 20/1) favours bacterial protein breakdown and conversion of (non-) protein N to NH_3 while higher C/N (above 25/1 to 30/1) favor incorporation of (non-) protein N into new microbial mass (Kutzner 2008, Ekinci *et al*. 2000).

In the midgut of housefly larvae, different amylases have been identified (Terra and Jordão 1989, Pimentel *et al*. 2018) indicating a capability to utilize carbohydrates including starch as an energy source. Housefly larvae have a complex trophic relationship with microorganisms and were found to ingest and digest microorganisms but it is not known what constituents of which species are essential (Espinosa-Fuentes and Terra 1987, Nayduch and Burrus 2019, Zurek *et al*. 2000). The current study hypothesized that housefly larvae reared on fresh chicken excreta compete with microorganisms for easily digestible carbohydrates. This hypothesis was tested experimentally by addition of easily digestible carbohydrates that can be used by both larvae and microorganisms (gelatinized starch, fructo-oligosaccharides (FOS)) to sterilised and unsterilised fresh chicken excreta. The effect of sterilisation and carbohydrate additions on larval performance (survival, growth) and substrate parameters (DM, N, uric acid, carbon, NH_3 content) were determined.

Materials and methods

Housefly culture

A housefly culture was established at Wageningen University & Research in January 2019, using the Dutch GK strain provided by Groningen University. Adult flies were kept in mesh Bugdorm™ cages (47.5×47.5×93 cm) at 1000-3000 per cage densities in a climate room (27 °C., 60% RH, 16:8 light-darkness) and fed ad libitum with a 50/50 mixture (v/v) of full-cream milk powder (De Zuidmolen, Groesbeek, the Netherlands) and deactivated yeast (BioToday, Ulvenhout, the Netherlands) provided in a thin layer in plastic plates. Pure sucrose was supplied separately as sugar cubes (Albert Heijn home brand, Zaandam, the Netherlands) in flat plastic plates. Tap water was supplied in polypropylene pots with cotton wicks inserted through the lids. Fresh food and water were provided every 48 hours. Larvae were reared in the same room in 1 L food grade polypropylene trays with mesh lids using a bran-based substrate: coarse wheat bran (Meneba, Rotterdam, the Netherlands), wheat flour (Albert Heijn home brand, Zaanstad, the Netherlands), milk powder, deactivated yeast and tap water in weight (as-is) ratio of 100:15:10:10:120, respectively. The population was sustained by allowing flies to oviposit on wheat bran substrate and partitioning the substrate and young larvae into the 1 L trays at 400-500 larvae to 300 g fresh substrate. After pupation, trays with pupae were placed in clean cages for eclosion.

Experimental substrates

Fresh (<24 h old) excreta of layers fed a commercial soy-based feed were obtained from Rondeel farm at Barneveld (the Netherlands). Portions (~400 g) were either directly frozen at -20 °C (E) or directly sterilized for 30 min in an autoclave at 121 °C (core temperature) and frozen (sE). Before use, frozen excreta were allowed to thaw overnight at 5 °C. Thawed 400 g portions were sampled for chemical analyses, after which 100 g was used as-is, or mixed with either 32 g gelatinized corn starch (Research Diet Services, Wijk bij Duurstede, the Netherlands) or 32 g fructo-oligosaccharides (Lamberts Healthcare, Kent, United Kingdom). To samples with additions, 50 mL tap water was also added to set moisture at ~33%. The added quantities were chosen to increase the C/N of pure excreta from 9/1 to approximately 20/1. Substrates were individually mixed by hand in 770 mL transparent polypropylene pots (PP Joni bekers, art. 003829, Gédé verpakkingen, Limmen, the Netherlands) fitted with a mesh Bugdorm™ lid. Six replicates were made per substrate. Substrate composition and chemical characteristics are provided in Table 1.

Table 1. Ingredient and chemical composition of chicken excreta substrates.

Item	Unsterilized chicken excreta			Sterilized chicken excreta		
	None	**Starch**	**FOS**	**None**	**Starch**	**FOS**
Ingredient composition (% as is)						
Excreta	100	55	55	100	55	55
Starch	0	18	0	0	18	0
FOS	0	0	18	0	0	18
Water	0	27	27	0	27	27
Chemical composition[1] (% DM)						
DM (% as is)	29.7	33.0	33.	32.4	34.5	34.5
Starch	1.7	51.6	0.8	1.9	50.2	1.0
Nitrogen	4.0	2.0	2.0	4.1	2.1	2.1
Uric acid	5.3	2.6	2.5	2.8	1.5	1.5
NH_3	0.3	-	-	0.2	--	-
NDF	36.7	20.1	20.1	34.1	18.9	18.9
ADF	21.1	3.7	3.7	20.4	3.6	3.6
C/N	9.3	21.3	20.1	9.0	19.8	18.7

FOS, fructo-oligosaccharides; DM, dry matter; NH_3, ammonia; NDF, neutral detergent fibre; ADF, acid detergent fibre; C/N, carbon to nitrogen ratio. [1] Nitrogen, uric acid, starch, NDF and ADF were analysed for sterilised and unsterilised excreta and calculated for the other substrates based on inclusion level.

Egg collection and partitioning

Oviposition pots were made by fitting black cotton socks, cut to 20% length, with the tip down into 300 ml round polypropylene pots which were half filled with semi-skimmed milk (Albert Heijn home brand, Zaandam, the Netherlands) with the tip of the sock submersed in the milk and covered with an aluminium foil lid containing a slit at the container edge to allow access. Oviposition pots were placed in cages with adult flies (7 to 10 days post eclosion) and left for 4-6 h. After removal, pots were kept in a larger closed container lined with wet (tap water) paper tissue to maintain high air humidity. Portions of eggs (~75 mg, ~1250 eggs), were deposited on the experimental substrates, in a crevice close to the container wall directly after weighing and lightly spraying (< 0.5 mL) with demineralized water. Inoculated substrates were placed on shelves in the same climate room as the adult cages for 5 days.

Harvesting, sampling and measuring of larvae and substrates

During the 5-day larval growth period, temperature of the inoculated substrates was measured daily using a probe thermometer (TFA Dostmann, Wertheim-Reicholzheim, Germany). On day 5, all inoculated substrates were placed on melting ice to slow down larval and microbial metabolism. Portions (20-30 g) of left-over inoculated substrates (substrate residue) without larvae were stored in polypropylene pots at -20 °C before freeze drying and chemical analysis. Larvae were quantitatively collected from the remaining substrate residues by floatation. This involved submerging the substrate residue in 2 L 14% NaCl solution at room temperature under gentle stirring, with difference in buoyancy causing larvae to float and substrate residue to sink. Floating larvae were collected from the top of the solution using a fine sieve, rinsed with tap water, transferred to filter paper to absorb excess water. All larvae were transferred to small polypropylene pots before being frozen at -20 °C. For determination of total and individual larval weight, larvae were thawed and evenly spread out on clean filter paper. After removal of remaining substrate particles, 30 larvae were randomly selected (by selection along a line from the operator to the far side of the paper) and individually weighed. Total wet larvae mass was determined by weighing all collected larvae of a single substrate residue together, including individually weighed larvae. After weighing larvae were frozen again at -20 °C for freeze drying and chemical analyses.

Chemical analyses

Ammonia and pH were determined in freshly thawed substrate residues without larvae. Ammonia was determined using a colorimetric method after deproteinisation with trichloroacetic acid (Weatherburn 1967). Uric acid was analyzed enzymatic-colorimetric using a commercial test kit (10694, Human GmbH, Wiesbaden, Germany). Analysis of total N and total C (Dumas method, ISO 16634-1, 2008), DM (ISO 6496, 1999), uric acid, starch (ISO 15914, 2004) and ADF/NDF content (ISO 13906, 2008) was performed on freeze dried material. All chemical analysis were performed in duplo, except for DM performed in simplo.

Calculations and statistical analyses

To partition egg portions by weight, egg count was derived from egg weight (individual egg weight: 0.06 mg) as egg weight was found to be highly uniform (mean ± SD: 0.06 ± 0.004 mg) in earlier tests weighing 8 clumps of 100-300 eggs followed by accurate counting. The number of larvae per replicate was estimated from the total wet larvae mass and individual larvae weights using a generalized linear model with a gamma distribution with log-link. Distributions of individual larval weight were bound by 0 and in many cases strongly skewed, making a gamma distribution (with log link) the best approximation to the density function. Model estimations of the 95% confidence intervals were checked by transforming histograms of individual larvae weights per sample (weight class width 0.5 mg) to fractions of the summed individual weights per sample with counts following from summation of weights per weight class divided by weight class means. Minimum and maximum average counts derived from histograms were highly similar to the 95% confidence interval limits generated by the model (all correlations > 0.99).

Larval survival was defined as the number of inoculation eggs divided by the mean number of larvae harvested. Testing for differences in survival between substrates was conducted using a generalized linear model (binomial distribution - logit link) with substrate replicate as experimental unit followed by pairwise tests (Tukey adjustment multiple comparisons).

Testing for differences in means with normally distributed error (total wet larvae mass, larval DM, C/N, pH) was achieved by a linear model (ANOVA). For individual larval weight, a generalized linear mixed model was used (gamma - log link, substrate replicate as random intercept), followed by pairwise tests. Continuous fractions with means close to 0 (bioconversion%, NH_3 content, uric acid content) were tested for differences using a generalized linear model (beta - logit link) again followed by pairwise tests. Tukey adjustment for multiple comparison was used in all pairwise testing. For all models, model residuals were visually inspected for normality and homoscedasticity and found to adhere sufficiently to the assumptions of the statistical tests. Analysis was conducted in R (R Core Team 2019).

Results

Characteristics of fresh substrates

Both unsterilised excreta (E) and sterilised excreta (sE) had a consistency like clay, making it difficult to produce homogeneous substrates. Although the same amounts of starch and FOS were added, their effect on the substrate were very different. Starch behaved as a solid while FOS dissolved in the added water, resulting in a more liquid consistency. Sterilization led to measurable changes in the chemical composition of the manure (Table 1). In sE, the DM and N content were slightly increased, leading to a slightly lower C/N. In sE, the uric acid content was almost halved, the NH_3 content decreased by 32% while NDF and ADF decreased slightly compared to E. Both E and sE had an initial pH of 7.1.

Larval performance on different substrates

Weighing 75 mg of eggs proved to be challenging as eggs stuck together and to the metal equipment used. A wet toothpick was found to be ideal to manipulate eggs and enable counting and weighing. The number of eggs used for inoculation of the substrates varied from 1228 to 1286, with no marked differences in number between substrates.

Larval performance differed between substrates (Table 2). Survival of larvae was highest, around 70%, in excreta without additions, E and sE. Survival was considerably lower for the other substrates, decreasing in the order: sE + star (57%), E + star (34%), E + FOS (9%) and sE + FOS (8%). Though subject to a lower survival, larvae grown in the sE + starch substrate were substantially heavier (13.2 mg) than those from the other substrates. Larvae from E (8.1 mg), E + star (7.5 mg) and sE (6.7 mg) were similar in size. E + FOS and sE + FOS resulted in the lightest larvae, 2.9 mg and 1.7 mg respectively. Larval dry matter content was lowest for sE and E, around 140 g/kg, and significantly higher for the carbohydrate additions. Furthermore, the distribution of individual larval weight differed between substrates (Figure S1). In E, sE and sE + star, distributions were normal or close to normal, whereas in E + star, E + FOS and sE + FOS distributions were (strong) left skewed, indicating that the majority of the larvae were lighter than the substrate mean.

Table 2. Count, survival, individual wet weight, total wet weight, dry matter content and C/N of housefly larvae reared on unsterilized (E) and sterilized (sE) chicken excreta substrates with or without starch (star) or fructo-oligosaccharides (FOS) addition.

Parameter	**Substrate**					
	E	**E + star**	**E + FOS**	**sE**	**sE + star**	**sE + FOS**
Count[1]	898 (836-965)	356 (281-451)	106 (80-141)	886 (802-978)	736 (669-807)	100 (80-125)
Survival[2] (%)	70 ± 3^{a}	34 ± 5^{c}	9 ± 4^{d}	70 ± 5^{a}	57 ± 8^{b}	8 ± 9^{d}
Individual wet weight (mg)[3]	8.1 ± 1.5^{b}	7.5 ± 4.9^{b}	2.9 ± 2.4^{c}	6.7 ± 1.8^{b}	13.2 ± 3.5^{a}	1.7 ± 1.0^{d}
Total larval wet weight (g)	7.2 ± 0.2^{b}	3.3 ± 0.5^{c}	0.3 ± 0.2^{d}	5.9 ± 0.9^{b}	9.7 ± 2.1^{a}	0.2 ± 0.2^{d}
Dry matter (g/kg)	144.1 ± 7.4^{b}	186.1 ± 12.3^{a}	193.7 ± 38.5^{a}	140.7 ± 4.5^{b}	209.8 ± 6.6^{a}	256.9 ± 29.3^{a}
C/N	4.4 ± 0.1^{c}	7.1 ± 0.4^{b}	6.1 ± 0.7^{b}	4.5 ± 0.1^{c}	8.1 ± 0.2^{a}	6.8 ± 0.1^{b}

Data presented as mean ± SD unless otherwise indicated. Means with different superscript letters within row differ with $p \leq 0.05$. [1] Data presented as mean and 95% confidence interval derived from a gamma distribution. [2] Mean and SD are based on estimated mean number of larvae divided by estimated number eggs used for inoculation. [3] Mean and SD are based on 25-30 larvae per substrate replicate (n = 6).

Substrate residues and bioconversion

Bioconversion of dry matter, C and N in to larval biomass differed between substrates (Table 3). The highest dry matter conversion (3.5, 2.6 and 3.1%) were recorded for E, sE and sE + star, respectively, with lower conversion for E + star (1.0%) and minimal for E + FOS and sE + FOS (both 0.1%). Nitrogen conversion was highest for sE + star (9.9%), followed by E (9.3%), sE (6.6%), E + star (4.0%) while E + FOS and sE + FOS had conversions close to 0. Carbon conversion was highest for E and sE + star (4.1 and 3.7%, respectively), lower for sE (3.1%) and E + star (1.0) with E + FOS and sE + FOS showing conversions close to 0.

Table 3. Mass of dry matter, nitrogen and carbon of housefly larvae reared on unsterilized (E) and sterilized (sE) chicken excreta substrates with or without starch (star) or fructo-oligosaccharides (FOS) addition and their bioconversion efficiency (BE).

Parameter	Substrate					
	E	**E + star**	**E + FOS**	**sE**	**sE + star**[1]	**sE + FOS**[2]
Dry matter						
Substrate (g)	29.7 ± 0.1	60.2 ± 0.1	60.2 ± 0.1	32.4 ± 0.1	62.9 ± 0.1	63.0 ± 0.1
Larvae (g)	1.0 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	0.8 ± 0.1	1.9 ± 0.4	0.1 ± 0.1
BE (%)	3.5 ± 0.3^{a}	1.0 ± 0.2^{c}	0.1 ± 0.1^{d}	2.6 ± 0.4^{b}	3.1 ± 0.6^{ab}	0.1 ± 0.1^{d}
Nitrogen						
Substrate (g)	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0
Larvae (g)	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
BE (%)	9.3 ± 0.6^{a}	4.0 ± 0.6^{c}	0.5 ± 0.3^{d}	6.6 ± 1.1^{b}	9.9 ± 2.0^{a}	0.5 ± 0.3^{d}
Carbon						
Substrate (g)	11.3 ± 0.0	25.5 ± 0.0	24.1 ± 0.0	12.1 ± 0.0	26.4 ± 0.0	25.0 ± 0.0
Larvae (g)	0.5 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	1.0 ± 0.2	0.0 ± 0.0
BE (%)	4.1 ± 0.3^{a}	1.0 ± 0.5^{b}	0.1 ± 0.1^{c}	3.0 ± 0.5^{d}	3.7 ± 0.8^{ad}	0.2 ± 0.1^{c}

Data presented as mean ± SD based on n = 6 unless otherwise indicated. Means with different superscript letters within row differ with $p \leq 0.05$. [1] Based on n = 5. [2] Based on n = 3.

Uric acid, ammonia, pH and C/N ratio

Substrate residue uric acid and NH_3 concentrations, pH and C/N differed between the six substrates (Table 4). Compared to the fresh substrates (Table 1), a decrease in uric acid content was observed in substrate residues of E, E + star and sE (92, 27 and 45%, respectively) while E + FOS, sE + star and sE + FOS showed an increase in uric acid (24, 47 and 53%, respectively). The highest free NH_3 concentrations were found in E and sE (0.4% of DM) with E + FOS, sE + FOS, sE + star having lower and similar content (0.1%) and no NH_3 was found in E + star. The substrate residues were alkaline for E (8.2) and sE (8.5) and acidic for the others, pH decreasing from E + star (5.3), sE + FOS (4.8), sE + star (4.6) to E + FOS (4.4). When C/N of substrate residues is compared to fresh substrates (Table 1), E and sE showed an increase from 9 to 16 and 12, respectively, whereas E + star and sE + star showed a large decrease to 14 and 15. E + FOS showed a minor decrease to 18 and sE + FOS residues showed no change in C/N.

Table 4. Uric acid and ammonia concentration (% of DM), pH and C/N in the residue of unsterilized (E) and sterilized (sE) chicken excreta substrates with or without starch (star) or fructo-oligosaccharides (FOS) addition.

Parameter	Substrate					
	E	**E + star**	**E + FOS**	**sE**	**sE + star**[1]	**sE + FOS**
Uric acid	0.4 ± 0.2^{a}	1.9 ± 0.7^{b}	3.1 ± 0.6^{c}	1.6 ± 0.5^{b}	2.2 ± 0.3^{bcd}	2.3 ± 0.1^{c}
Ammonia	0.4 ± 0.1^{a}	0.0 ± 0.0^{b}	0.1 ± 0.0^{c}	0.4 ± 0.1^{a}	0.1 ± 0.0^{d}	0.1 ± 0.0^{c}
pH	8.2 ± 0.1^{a}	5.3 ± 0.4^{c}	4.4 ± 0.0^{d}	8.5 ± 0.2^{b}	4.6 ± 0.1^{ab}	4.8 ± 0.1^{d}
C/N	16.0 ± 1.1^{a}	14.2 ± 0.8^{ab}	17.8 ± 1.9^{ac}	12.4 ± 1.1^{bd}	15.3 ± 1.6^{ab}	18.3 ± 0.8^{c}

Data presented as mean ± SD based on n = 6 unless otherwise indicated. Means with different superscript letters within row differ with $p \leq 0.05$. [1] Based on n = 5.

Discussion

The chicken excreta was autoclaved for 30 min at 121 °C and 103 kPA, parameters that are commonly effective to inactivate the vast majority of microbiota including spores in various food and feed products. Sterilisation led to slight increases in DM and N content and a decrease in ammonia content. Though not statistically significant, larvae grown in sE were lighter and total larval mass lower than that of E. This difference could be caused by destruction of heat sensitive compounds essential to larvae, such as B vitamins. Differences in temperature profile of the inoculated substrates support lower microbial metabolism in the sterilized substrates (Figure S2). The temperature profile of E showed an initial rise of temperature during the first 24 to 48 h, similar to that of aerobic composting of fresh chicken manure (Kajiya *et al*. 2015, Hwang *et al*. 2020), whereas sE showed a later more distinct peak. In contrast, sE + star heated up faster than E + star, but the latter attained a higher temperature for a longer time. However, the difference in temperature profile could also be due to heat production of the larvae themselves, that had a considerably larger mass in sE + star. No reports of heat production by housefly larvae were found in the literature, but larval aggregations of Calliphorid species that are taxonomically related with and ecologically similar to the Muscid flies were found to be much warmer than ambient temperature (Anderson and van Laerhoven 1996). The pH of E + star and E + FOS was higher than their sterilized counterparts, which could indicate differential microbial activity as a result of sterilisation, the effect not being confounded for FOS due to the low number of larvae present. To distinguish the effects of sterilisation from those of larvae, it is of interest to include control substrates without larvae in future larval nutrition studies that involve substrates with a high microbial load.

Larval performance and bioconversion were much lower for FOS compared to the other substrates. Though water content was equivalent to the other substrates, adding FOS resulted in a much more viscous consistency compared to starch. The FOS substrates appeared dry during mixing, but turned to a more viscous consistency after several hours. The high viscosity appeared to prevent larvae from entering the substrates, as larvae were only observed on the surface, where for the other substrates larvae were observed throughout the material after 5 days of larval growth. The viscosity possibly limited the availability of oxygen, which not only limited the larvae burrowing but is also the probable cause for the absence of heat production of the FOS substrates (Fig. S2). The intended effect of FOS

addition was to provide carbohydrates, indigestible by endogenous enzymes of larvae but digestible by microorganisms such as *Bifidobacterium* sp. (Kelly 2008). The FOS-metabolizing microorganisms could subsequently be digested by the larvae, enhancing larval performance compared to the control (sE + FOS). The current study design (high dose in a moist environment) resulted in a negative effect of the FOS treatments. To investigate the effects of manipulation of microbiota by addition of FOS, future studies should not only control moisture levels but also control substrate consistency.

Sterilisation improved larval performance of the chicken excreta with starch. Average wet yield increased (3.3 vs 9.7 g) as did the average larval weight (7.5 vs 13.2 mg). These results support the hypothesis that larvae and microbiota compete for carbohydrates. The contrast between E + star, E and sE also supports the study hypothesis. The daily temperature profile (Fig. S2) shows that E + star reached a higher temperature and produced heat later and longer than the other substrates. Aerobic decomposition is known for its phases of heat production by microorganisms in relation to the availability of metabolizable nitrogen and carbon (Kutzner 2008). If enough metabolizable carbon is present, microbiota (i.e. *Bacillus*, *Pseudomonas*, *Bifidobacterium*, *Candida* spp.) generate heat by oxidizing the carbon while incorporating N into microbial biomass. This process could very well have a negative impact on larval nutrition if larvae are dependent on available free protein or on specific nutrients provided by microbiota associated with low C/N substrates, that are depleted when easily digestible carbohydrates are abundant.

Leaving the larval survival of <10% in substrates with FOS out of consideration, survival was high (70%) in E and sE, lower in sE + star (57%) and lowest in E + star (34%). These survival rates are comparable to those found in literature, 40 to 60% for fresh pig feces with variable amounts of sawdust and 40 to 85% for pure chicken excreta at larval densities of 3 to 6 larvae per g substrate (Calvert *et al*. 1970, Barnard *et al*. 1998, Cickova *et al*. 2012). However, studies are difficult to compare as studies differ in terms of manure quantities, larval densities and definitions of survival (i.e. eggs to pupae, eggs to adults, larvae to pupae). It is not known which age group was most affected by mortality but, when not related to differences in mechanical properties of the substrates, the differential survival found might also indicate a nutritional dependency of larvae for free protein or microbiota associated with high protein (low C/N) substrates.

Individual larval mass distribution differed between substrates (Figure S1), with E, sE and sE + star showing an approximately normal or symmetric distribution, while the distributions in E + star and the FOS substrates were left skewed (relatively more small individuals). This might also be an indication of larval competition for nutrients or microclimates as (animal) body size tends to be inversely related to competition strength with relatively few large individuals suppressing nutrient intake of relatively numerous small ones (see for example Ward *et al*. 2006). But this relation in (housefly) larvae could be complicated by the existence of Allee effects, high densities of larvae feeding more efficiently than low densities, as found for Drosophila, or cannibalism (Wertheim *et al*. 2002, Lam *et al*. 2007). Additional research using different densities of larvae is needed to investigate the relation between individual larva weight, competition for food and total larvae mass.

The DM values for larvae grown on E and sE are low compared to the values found for larvae reported in literature of 240 to 250 g/kg of larvae grown in a mixture of layer manure and food waste (Van Zanten *et al*. 2015) and 300 g/kg of larvae grown in fresh dairy cattle feces (Hussein *et al*. 2017). Larvae reared in E and sE had a C/N of 4.5 while larvae from E + star and sE + star had ratios of 7.1 and 8.1, respectively and larvae from E + FOS and sE + FOS ratios of 6.1 and 6.8, respectively. Adding starch clearly increased the C/N, potentially due to larvae being fatter or the composition of the gut content.

In this study, larval bioconversion of DM, N and C was calculated. Bioconversion of the DM and N fraction are important determinants for the potential of bioconversion with insects in order to minimize waste and maximize N deposition while preventing N emission. Excluding the FOS substrates, the highest bioconversion of DM was found for E (3.5%), followed by sE + star (3.1%), sE (2.6%) and E + star (1.0%). The observed DM bioconversions of E and sE are low in the range found in literature for poultry manure (1.1-10.2%) (Calvert *et al*. 1970, Barnard *et al*. 1998, Zi-zhe *et al*. 2017). The difficulty in comparing results with data in the literature is that other studies were conducted with different densities of larvae and different amounts of manure. The study closest in terms of density to the present study is the one of Barnard *et al*. (1998) who used 3 larvae per g in 100 g fresh to 2 week old layer manure and found a DM bioconversion of 2.4%. The observed DM conversion of E is similar to that of black soldier fly larvae (*Hermetia illucens*) reared on chicken manure, with values found of 3.4% by Oonincx *et al*. (2015) but lower than the 9.8% found by Rehman *et al*. (2017).

Data on N bioconversion by housefly larvae are not available in the literature, this study being the first to report N bioconversion. The highest N bioconversion was found for sE + star and E (9.9 and 9.3%), lower for sE (6.6%) and E + star (4%) and only 0.5% for the FOS substrates. Interestingly, while sE + star (9.9%) proved to be the best of the substrates for N deposition into larvae, N bioconversion was also high for pure E (9.3%). During the larval growth period, the E + star and sE + star treatments were observed to have little to no NH_3 odour while both E and sE substrates emitted a strong NH_3 odor during the larval growth period, indicating NH_3 emission. The high NH_3 emission compared to sE + star did not seem to affect larval N deposition. This high N bioconversion of pure excreta might again be an indication of larval specialization to a protein diet or interaction with specific microorganisms that play a role in larval N utilization. The observed low N bioconversion of E + star could be caused not only by fixation of N into microbial biomass but also by changing the microbial species composition rendering the substrate unfit for (early stage) larval growth. The N bioconversion for excreta found in this study is much higher than the 4.6% reported for black soldier flies by Oonincx *et al*. (2015). This indicates that housefly larvae have bioconversion potential for nitrogenous material and efficient upcycling of N.

Conclusions

This study supports the hypothesis that housefly larvae compete with microorganisms for an easily digestible carbohydrate (starch) in fresh chicken excreta. Heat sterilized chicken excreta alone led to lighter larvae and lower total larval mass compared to unsterilized chicken excreta, whereas sterilized chicken excreta with added starch (50% of substrate DM) led to heavier larvae and a much higher total larval mass while showing a lower survival compared to unsterilized chicken excreta with added starch. Addition of fructo-oligosaccharides (50% of substrate DM) to both sterilized and unsterilized excreta resulted in minimal growth and total biomass of larvae, attributable to the high viscosity of the substrate. Different substrates showed different temperature profiles during larval growth but effects of microorganisms and larvae on temperature cannot be separated with the used experimental design. The size distribution of individual larvae differed markedly between experimental substrates, which might be an indication of nutritional status. The found bioconversion efficiencies for DM are on the low side of the range found in literature, while the bioconversion efficiency of N is reported for the first time in this study. N bioconversion efficiency is relatively high, around 9%, for both unsterilized excreta and sterilized excreta with starch.

References

Alexandratos, N., Bruinsma, J. (2012). World agriculture towards 2030/2050: The 2012 revision (ESA Working Paper No. 12-03). Rome, Italy: Food and Agriculture Organization of the United Nations.

Anderson, G. S., van Laerhoven, S. L. (1996). Initial studies on insect succession on carrion in southwestern British Columbia. *Journal of Forensic Science*, 41, 617-625.

Apajalahti, J., Kettunen, A., Graham, H. (2004). Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Science Journal*, 60(2), 223-232.

Barnard, D. R., Harms, R. H., Sloan, D. R. (1998). Biodegradation of poultry manure by house fly (Diptera: Muscidae). *Environmental Entomology*, 27, 600-605.

Ben-Amor, K., Heilig, H., Smidt, H., Vaughan, E. E., Abee, T., de Vos, W. M. (2005). Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. *Applied and Environmental Microbiology*, 71(8), 4679-4689.

Calvert, C. C., Morgan, N. O., Martin, R. D. (1970). House fly larvae: Biodegradation of hen excreta to useful products. *Poultry Science*, 49(2), 588-589.

Cickova, H., Pastor, B., Kozanek, M., Martínez-Sánchez, A., Rojo, S., Takac, P. (2012). Biodegradation of pig manure by the housefly, *Musca domestica*: A viable ecological strategy for pig manure management. *PLoS ONE*, 7(3), e32798.

Chen, S., Wen, Z., Liao, W., Liu, C., Kincaid, R. L., Harrison, J. H., Elliot, D. C., Brown, M. D., Stevens, D. J. (2005). Studies into using manure in a biorefinery concept. In: Davison, B. H., Evans, B. R., Finkelstein, M., McMillan, J. D. (eds) Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals. Totowa, NJ, USA: Humana Press.

Ekinci, K., Keener, H. M., Elwell, D. L. (2000). Composting short paper fiber with broiler litter and additives. Part I: Effects of initial pH and carbon/nitrogen ratio on ammonia emission. *Compost Science and Utilization*, 8, 160-172.

Espinosa-Fuentes, F. P., Terra, W. R. (1987). Physiological adaptations for digestion bacteria. Water fluxes and distribution of digestive enzymes in *Musca domestica* larval midgut. *Insect Biochemistry and Molecular Biology*, 17, 809-817.

FAOSTAT (2020). FAO statistical database. Retrieved February 2021. Available at: http://www.fao.org/poultry-production-products/en/

Gadzama, I. U., Ndudim, R. K. (2019). Nutritional composition of housefly larvae meal: a sustainable protein source for animal production - a review. *Acta Scientific Agriculture*, 3, 74-77.

Godfray, H. C. J., Aveyard, P., Garnett, T., Hall, J. W., Key, T. J., Lorimer, J., Pierrehumbert, P. S., Springmann, M., Jebb, S. A. (2018). Meat consumption, health, and the environment. *Science*, 361, 6399-7407.

Hashizume, A., Ido, A., Ohta, T., Thiaw, S. T., Morita, R., Nishikawa, M., Takahashi, T., Miura, C., Miura, T. (2019). Housefly (*Musca domestica*) larvae preparations after removing the hydrophobic fraction are effective alternatives to fish meal in aquaculture feed for red seabream (*Pagrus major*). *Fishes*, 4, 38.

Henchion, M., Hayes, M., Mullen, A. M., Fenelon, M., Tiwari, B. (2017). Future protein supply and demand: Strategies and factors influencing a sustainable equilibrium. *Foods*, 6(7), 53-71.

Hussein, M., Pillai, V. V., Goddard, J. M., Park, H. G., Kothapalli, K. S., Ross, D. A., Selvaraj, V. (2017). Sustainable production of housefly (*Musca domestica*) larvae as a protein-rich feed ingredient by utilizing cattle manure. *PLoS ONE*, 12(2), e0171708.

Hwang, H. Y., Kim, S. H., Kim, M. S., Park, S. J., Lee, C. H. (2020). Co-composting of chicken manure with organic wastes: characterization of gases emissions and compost quality. *Applied Biological Chemistry*, 63(1), 2-10.

ISO 15914 (2004). Animal feeding stuffs - determination of total starch content. International Organization for Standardization, Geneva, Switzerland.

ISO 6496 (1999). Animal feeding stuffs - determination of moisture and other volatile matter content. International Organization for Standardization, Geneva, Switzerland.

ISO 16634-1 (2008). Food products - determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content - Part 1: Oilseeds and animal feeding stuffs. International Organization for Standardization, Geneva, Switzerland.

ISO 13906 (2008). Animal feeding stuffs - determination of acid detergent fibre (ADF) and acid detergent lignin (ADL) contents. International Organization for Standardization, Geneva, Switzerland.

Kajiya, Y., Ishii, Y., Fukuyama, K., Idota, S. (2015). Precise optimization of poultry manure composting using an experimental small-scale apparatus. *Journal of Biological Sciences*, 15(1), 40-44.

Kelly, G. (2008). Inulin-type prebiotics - a review: Part 1. *Alternative Medicine Review*, 13(4), 315-329.

Kurbanoglu, E., Algur, O. (2002). Single-cell protein production from ram horn hydrolysate by bacteria. *Bioresource Technology*, 85(2), 125-129.

Kutzner, H. J. (2008). Microbiology of composting. In Biotechnology Set, 2nd ed. Berlin, Germany: Wiley-VCH Verlag GmbH.

Lam, K., Babor, D., Duthie, B., Babor, E., Moore, M., Gries, G. (2007). Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. *Animal Behaviour*, 74(1), 81-92.

Maeda, K., Hanajima, D., Toyoda, S., Yoshida, N., Morioka, R., Osada, T. (2011). Microbiology of nitrogen cycle in animal manure compost: nitrogen cycle in manure compost. *Microbial Biotechnology*, 4(6), 700-709.

Metcalf, L., Eddy, H. P., Tchobanoglous, G. (2004). Wastewater Engineering: Treatment, Disposal, and Reuse (4th ed.). McGraw-Hill Series in Water Resources and Environmental Engineering. New York, USA: McGraw-Hill, 920 pp.

Miller, B. F., Teotia, J. S., Thatcher, T. O. (1974). Digestion of poultry manure by *Musca domestica*. *British Poultry Science*, 15(2), 231-234.

Nahm, K. (2003). Evaluation of the nitrogen content in poultry manure. *World's Poultry Science Journal*, 59(1), 77-88.

Nayduch, D., Burrus, R. G. (2019). Flourishing in filth: House fly-microbe interactions across life history. *Annals of the Entomological Society of America*, 110, 18-26.

Nicholson, F. A., Groves, S. J., Chambers, B. J. (2005). Pathogen survival during livestock manure storage and following land application. *Bioresource Technology*, 96, 135-143.

Nodar, R., Acea, M., Carballas, T. (1990). Microbial composition of poultry excreta. *Biological Wastes*, 33(2), 95-105.

Ogunji, J., Toor, R. U. A. S., Schulz, C., Kloas, W. (2008). Growth performance, nutrient utilization of Nile tilapia (*Oreochromis niloticus*) fed housefly maggot meal (magmeal) diets. *Turkish Journal of Fisheries and Aquatic Sciences*, 8(1), 141-147.

Oonincx, D. G. A. B., van Huis, A., van Loon, J. J. A. (2015). Nutrient utilisation by black soldier flies fed with chicken, pig, or cow manure. *Journal of Insects as Food and Feed*, 1(2), 131-139.

Pieterse, E., Pretorius, Q. (2014). Nutritional evaluation of dried larvae and pupae meal of the housefly (*Musca domestica*) using chemical and broiler-based biological assays. *Animal Production Science*, 54(3), 347-355.

Pimentel, A. C., Barroso, I. C., Ferreira, J. M. J., Dias, R. O., Ferreira, C., Terra, W. R. (2018). Molecular machinery of starch digestion and glucose absorption along the midgut of *Musca domestica*. *Journal of Insect Physiology*, 109, 11-20.

Pretorius, Q. (2011). The evaluation of larvae of *Musca domestica* (common house fly) as protein source for broiler production. M.Sc. thesis, Agricultural Animal Sciences, University of Stellenbosch, Stellenbosch, South Africa.

R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rehman, K. U., Cai, M., Xiao, X., Zheng, L., Wang, H., Soomro, A. A., Zhou, Y., Li, W., Yu, Z., Zhang, J. (2017). Cellulose decomposition and larval biomass production from the co-digestion of dairy manure and chicken manure by mini-livestock (*Hermetia illucens* L.). *Journal of Environmental Management*, 196, 458-465.

Ritala, A., Häkkinen, S., Toivari, M., Wiebe, M. (2017). Single cell protein - state-of-the-art, industrial landscape and patents 2001-2016. *Frontiers in Microbiology*, 8, Article 2009.

Sekar, K., Linker, S., Nguyen, J., Grünhagen, A., Stocker, R., Sauer, U. (2020). Bacterial glycogen provides short-term benefits in changing environments. *Applied and Environmental Microbiology*, 86(9), e0049-20.

Statista (2020). Number of chickens worldwide from 1990 to 2018. Statista. Retrieved February 23, 2021. https://www.statista.com/statistics/263962/

Steger, K., Eklind, Y., Olsson, J., Sundh, I. (2005). Microbial community growth and utilization of carbon constituents during thermophilic composting at different oxygen levels. *Microbial Ecology*, 50(2), 163-171.

Terra, W., Jordão, B. (1989). Final digestion of starch in *Musca domestica* larvae: Distribution and properties of midgut α-D-glucosidases and glucoamylase. *Insect Biochemistry*, 19(3), 285-292.

van Zanten, H. H. E., Mollenhorst, H., Oonincx, D. G. A. B., Bikker, P., Meerburg, B. G., de Boer, I. J. M. (2015). From environmental nuisance to environmental opportunity: Housefly larvae convert waste to livestock feed. *Journal of Cleaner Production*, 102, 362-369.

Veldkamp, T., van Duinkerken, G., van Huis, A., Lakemond, C. M. M., Ottevanger, E., Bosch, G., van Boekel, M. A. J. S. (2012). Insects as a sustainable feed ingredient in pig and poultry diets: A feasibility study. Report 638, Wageningen UR Livestock Research.

Wang, K., Li, W., Li, X., Ren, N. (2015). Spatial nitrifications of microbial processes during composting of swine, cow and chicken manure. *Scientific Reports*, 5, 14932.

Ward, A. J. W., Webster, M. M., Hart, P. J. B. (2006). Intraspecific food competition in fishes. *Fish and Fisheries*, 7(4), 231-261.

Weatherburn, M.W. (1967). Phenol hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, 39, 971-974.

Wertheim, B., Dicke, M., Vet, L. E. M. (2002). Behavioural plasticity in support of a benefit for aggregation pheromone use in *Drosophila melanogaster*. *Entomologia Experimentalis et Applicata*, 103(1), 61-71.

Weurding, R. E., Veldman, A., Veen, W. A. G., van Aar, P. J., Verstegen, M. W. A. (2001). Starch digestion in the small intestine of broiler chickens differs among feedstuffs. *Journal of Nutrition*, 131(9), 2329-2335.

Wilson, W., Roach, P., Montero, M., Baroja-Fernández, E., Muñoz, F., Eydallin, G., Viale, A. M., Pozueta-Romero, J. (2010). Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiology Reviews*, 34(6), 952-985.

Zi-zhe, A., De-Po, Y., Sheng-Qing, W., Yong, W., Reaney, M. J. T., Zhi-Min, Z., Long-Ping, Z., Guo, S., Yi, N., Dong, Z., Hui-Ran, N., Wen-Zhe, Y. (2017). Conversion of poultry manure to biodiesel: A practical method of producing fatty acid methyl esters via housefly larval lipid. *Fuel*, 210, 463-471.

Zhang, Z., Shen, J., Wang, H., Liu, M., Wu, L., Ping, F., He, Q., Li, H., Zheng, C., Xu, X. (2014). Attenuation of veterinary antibiotics in full-scale vermicomposting of swine manure via the housefly larvae (*Musca domestica*). *Scientific Reports*, 4, 6844.

Zurek, L., Schal, C., Watson, D. (2000). Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. *Journal of Medical Entomology*, 37(6), 924-928.

Supplementary material

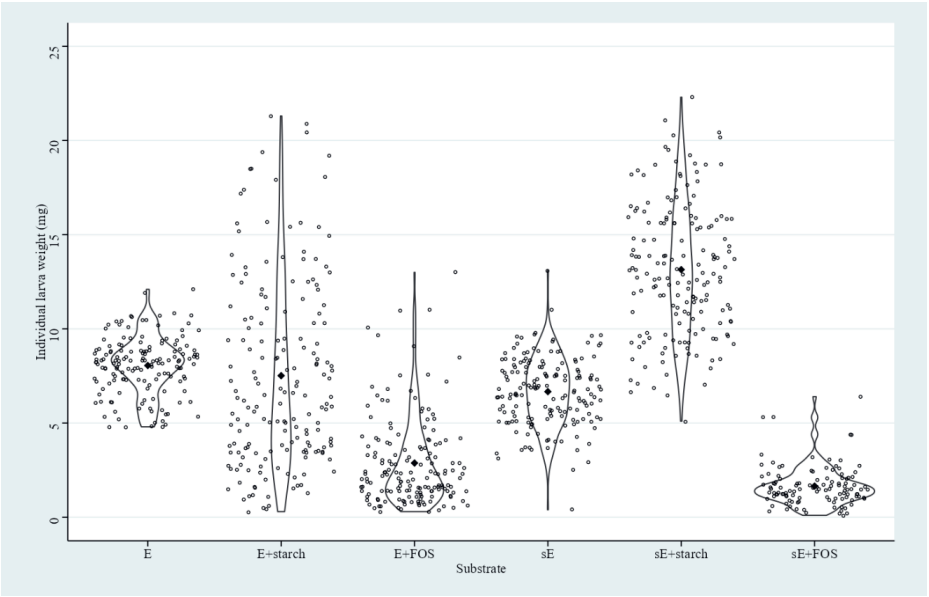


Figure S1. Violin plot of individual larva weight (n = 944) grown on unsterilized (E) and sterilized (sE) chicken excreta substrates with or without starch (star) or fructo-oligosaccharides (FOS) addition. Group means are represented by diamond shapes.

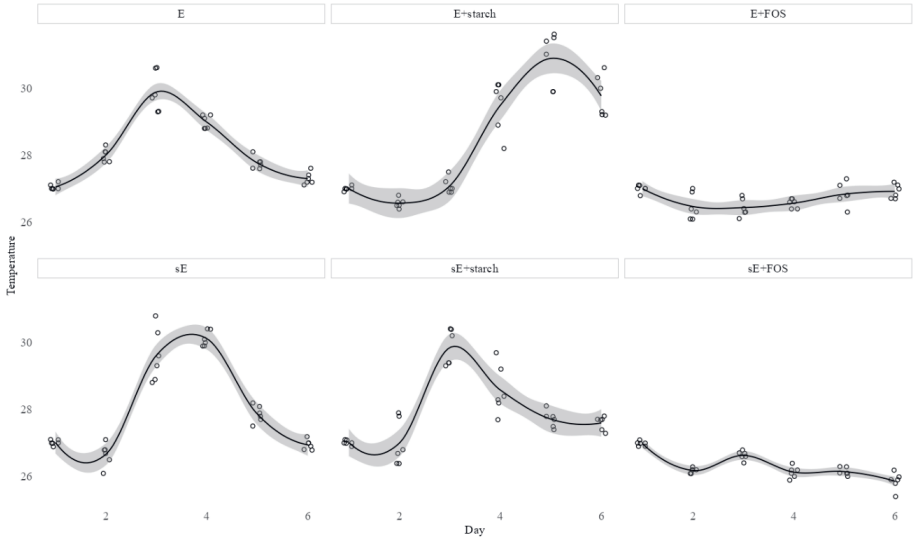


Figure S2. Substrate temperature per day for unsterilized (E) and sterilized (sE) chicken excreta substrates with or without starch (star) or fructo-oligosaccharides (FOS) addition. Solid lines are day means with the grey scale indicating the 95% confidence interval

.

Chapter 3

Dose-response effects of starch in sterilized and unsterilized chicken excreta on *Musca domestica* larval growth and bioconversion efficiency

L.W. Pisa, D.G.A.B. Oonincx, G. Bosch, W.H. Hendriks

Journal of Insects as Food and Feed (2024), 10(2), 447-464

Abstract

Housefly (*Musca domestica* L.) larvae can convert animal manures into valuable protein and fat but little is known about their nutritional requirements. This study assessed the effects of differential starch content in unsterilized (UE) and heat sterilized (SE) chicken excreta on larval performance and bioconversion. Gelatinized corn starch was added to excreta resulting in 11 diets with 0 to 50% starch of dry mass. Individual larval mass increased from (mean ± SD) 12 ± 0.2 (UE) and 13 ± 0.4 (SE) mg at 0% starch up to 18 ± 0.4 (UE) and 18 ± 0.5 (SE) mg at 15% starch and decreased to 5 ± 0.4 (UE and SE) mg in 50% starch. Non-normal distributions of individual larva mass were observed at higher starch concentrations. Larval wet yield showed a curvilinear relation with starch content, with maxima of 39 ± 2.3 g in UE at 10% starch, and 43 ± 2.8 g in SE at 15% starch. The decrease in yield after the maxima was faster in UE compared to SE. Larval survival was highest in 0% starch, 80 ± 8% (UE) and 72 ± 13% (SE), linearly decreasing to 14 ± 7% (UE) and 18 ± 3% (SE) in 50% starch. Dry matter bioconversion increased from 4 ± 0.1% (UE) and 4 ± 0.3% (SE) in 0% starch to 6 ± 0.2 in UE with 10% starch and 7 ± 0.3 in SE with 15% starch, decreasing to close to 0 in 50% starch. Highest nitrogen bioconversion was 15 ± 0.5% in UE with 10% starch and 15 ± 0.5% in SE with 15% starch. Optimal starch inclusions to UE and SE increased nitrogen bioconversion with 40 and 100% respectively, compared to pure excreta. Pronounced effects of starch on pH, uric acid, ammonia and diet residue C/N content were also observed. The results indicate that adding easily digestible energy to chicken excreta can substantially increase larval yield and nitrogen bioconversion while reducing ammonia content in the substrate residue.

Introduction

The housefly (*Musca domestica* L.) is a promising insect species for converting organic waste into protein, fat and chitin (Gadzama and Ndudim 2019, Sánchez-Muros *et al*. 2014, van Huis 2019). Housefly mass-rearing systems can potentially play a significant role in waste management and reduce the volume and undesirable characteristics of waste streams such as animal manures (Čičková *et al*. 2012). In addition, the use of larvae and derived products such as protein can contribute to circularization of agriculture, closing the loop by upcycling nutrients and increase the efficiency of plant-animal food systems (Čičková *et al*. 2015, Lalander and Vinnerås 2022).

In nature, housefly larvae develop in animal feces and carrion that are either fresh or at an early stage of decomposition. Although larvae can grow on cattle and other ruminant feces, survival, weight and adult weight are generally lower than when grown on omnivore or carnivore feces (Khan *et al*. 2012, Miranda *et al*. 2019, Shah *et al*. 2016). Optimal feces for larvae growth have two characteristics in common: a relatively low C/N (7 to 12) and a high protein content (33 to 58 mg/g free and protein bound amino acids) (Frank *et al*. 2007, Holter and Scholz 2007). The protein content predominantly originates from the large bacterial fraction found in feces, with bacterial dry matter consisting of 50 to 80% protein (Kurbanoglu and Algur, 2002, Ritala *et al*. 2017). Chickens are omnivores in nature with up to 60% of the nitrogen (N) in their excreta consisting in the form of uric acid due to the presence of a cloaca (Nahm, 2003). Uric acid easily decomposes to ammonia during storage or composting of chicken excreta, leading to high ammonia emissions that increase the environmental burden of chicken production (Murakami *et al*. 2011).

In general, fresh feces and excreta of various animal species contain little to no easily digestible carbohydrates, such as starch or glucose, as their ileal digestibility is high and if undigested (e.g. resistant starch, fructo-oligosaccharides) are quickly used by microbiota in the colon (Pluske *et al*. 2007, Roelof *et al*. 2001). Contradictory to this absence of easily digestible carbohydrates is the fact that starch digesting enzymes amylase, glucosidase and glucoamylase were found in the midgut of older housefly larvae (Hirashima *et al*.1989, Sinha 1975, Terra and Jordão 1989). The substrate for these enzymes is probably bacterial glycogen, as it can be synthesized by several bacteria species present in feces (Goh and Klaenhammer 2014, Wang *et al*. 2018). The capability of larvae to digest starch might be the reason for the effectivity of the Gainesville (laboratory) diet, where larvae grown on this high starch (15 to 28% of DM) wheat bran based substrate are heavier than larvae grown on animal feces (Hogsette 1992, Khan *et al*. 2012).

The amino acids in proteins ingested by larvae can be used for body protein synthesis (growth), glucose neogenesis and as an energy source via protein catabolism and oxidation. The latter is generally disadvantageous as the amino acids potentially to be used for growth are wasted with the excess nitrogen voided by housefly larvae as uric acid, leading to increased emission of ammonia (Weihrauch and O'Donnell 2021). As housefly larvae appear to be capable of utilizing starch, adding starch to chicken excreta could decrease gluconeogenesis and amino acid catabolism, thereby increasing larvae growth while reducing larval nitrogen excretion and improving excreta N bioconversion.

Fresh excreta have a high microbial load and adding starch can also be beneficial to microorganisms competing with larvae for other nutrients, thereby decreasing larval survival, growth and yield. In a previous experiment, adding gelatinized corn starch (~50% of DM) to heat sterilized chicken excreta increased average individual larva mass by ~80% and average total larva yield by ~200% compared to unsterilized excreta, resulting in an increased N bioconversion of ~ 150% (Pisa *et al*. 2022). Also, the addition of starch substantially decreased free ammonia in both sterilized and unsterilized diet residues (Pisa *et al*. 2022). The dose-response relation between the amount of starch in the larval diet and larval individual mass, total larval yield, larval survival and substrate bioconversion is unknown.

This study aimed to determine the optimal addition of gelatinized starch to both sterilized (initial microbiota killed) and unsterilized (initial microbiota alive) fresh chicken excreta for larval growth, larval yield and excreta bioconversion.

Materials and methods

Housefly culture

A housefly (*Musca domestica*) culture was established at Wageningen University & Research in October 2021 using larvae of the Dutch GK strain provided by Groningen University, the Netherlands. Adult flies were kept in mesh Bugdorm™ cages (47.5×47.5×93 cm) with 2000- 3000 individuals per cage in a climate room (27 °C., 60% RH, 16:8 light-darkness) and provided milk-based baby formula (0 to 6 months standard, Albert Heijn home brand, Zaandam, the Netherlands) ad libitum as a thin layer in disposable plastic plates. Sucrose cubes (Albert Heijn home brand, Zaandam, the Netherlands) were supplied separately in flat plastic plates. Tap water was provided in polypropylene pots with cotton wicks inserted through the lids. Fresh food and water were provided every 48 hours. Larvae were reared in the same climate room in 1 L food grade polypropylene trays with mesh lids using a substrate composed of a layer of soaked dog kibble (adult dog kibble, Albert Heijn home brand, Zaandam, the Netherlands), covered with dry wheat bran (Meneba, Rotterdam, the Netherlands), using a kibble, bran and water weight ratio of 1:1.5:2. The population was sustained by allowing flies to oviposit on fresh substrate and partitioning the resulting substrate with young larvae after 48 hours into new substrate trays at 400-500 larvae to ~300 g fresh substrate. After pupation, trays with pupae were placed in clean cages for eclosion.

Egg collection and partitioning

Housefly eggs were collected and partitioned by weight (8 eggs/g wet diet) into experimental substrates following the same procedure as described by Pisa *et al*. (2022).

Experimental substrates

Fresh (<24 h old) excreta of laying hens fed on a commercial soy-wheat based feed were obtained from Rondeel farm (Barneveld, the Netherlands) with portions (~400 g) either directly frozen at -20 °C (unsterilised excreta, UE) or directly sterilised for 30 min in an autoclave at 121 °C (core temperature) and then frozen at -20 °C (sterilised excreta, SE). Before use, frozen excreta were allowed to thaw overnight at 5 °C and homogenised using an FGC 10-2 bowl cutter (Feuma

Gastromaschinen GmbH, Gößnitz, Germany). Homogenisation was conducted to obtain a particulate, fluffy material which was easy to stir. During homogenisation, feathers and eggshells were manually removed and after homogenization a sample was taken for moisture content determination (5 g, n = 3) using a MB 120 moisture analyzer (OHAUS Europe GmbH, Nänikon, Switzerland). Larger representative samples (100 g, n = 3) were taken and stored at -20 °C until chemical analyses. Moisture content of gelatinized corn starch (Research Diet Services, Wijk bij Duurstede, the Netherlands) was also determined (5 g, n = 3) with the same moisture analyzer. Ingredients composition and diet composition are shown in Table 1 and 2, respectively.

Table 1. Analyzed composition (g/kg dry matter) of unsterilised and sterilised chicken excreta and starch.

Item[1]	Chicken excreta		Starch
	Unsterilized	Sterilized	
Dry matter (g/kg as is)	444 ± 8.0	446 ± 8.0	908 ± 2.1
Starch	0.9 ± 0.1	1.0 ± 0.5	894.1 ± 4.0
Ammonia	5.6 ± 0.1	4.9 ± 0.1	NA[2]
Uric acid	48.9 ± 4.1	37.5 ± 0.5	NA[2]
Total carbon	347 ± 9.0	350 ± 9.9	399 ± 0.8
Total nitrogen	38.9 ± 1.4	39.1 ± 1.3	1.0 ± 0.0

[1]Values are average ± standard deviation, n = 3. [2]Not analyzed.

Table 2. Ingredient composition and calculated chemical compositions of sterilized (SE) and unsterilized (UE) chicken excreta-starch diets.

Item	Formulated starch content (%)										
	0	5	10	15	20	25	30	35	40	45	50
Ingredient composition (g)											
Excreta	300	285	270	255	240	225	210	195	180	165	150
Starch	0	7.5	15	22.5	30	37.5	45	52.5	60	67.5	75
Water	142.0	149.3	156.5	164.8	173	179.3	187.5	194.8	202	210.3	217.5
Chemical composition (g/kg dry matter)											
Dry matter (g/kg as is)	300	300	300	300	300	300	300	300	300	300	300
Starch	0	50	100	150	200	250	300	350	400	450	500
Ammonia-UE	5.6	5.3	5.0	4.8	4.5	4.2	3.9	3.6	3.4	3.1	2.8
Ammonia-SE	4.9	4.7	4.4	4.2	3.9	3.7	3.4	3.2	2.9	2.7	2.5
Uric acid-UE	48.9	46.5	44.0	41.6	39.1	36.7	34.2	31.8	29.3	26.9	24.5
Uric acid-SE	37.5	35.6	33.8	31.9	30.0	28.1	26.3	24.4	22.5	20.6	18.8
pH-UE	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7	8.7
pH-SE	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
Total carbon	347	350	353	355	358	361	363	366	368	371	374
Total nitrogen	39.0	37.1	35.1	33.2	31.2	29.3	27.3	25.4	23.4	21.5	19.5
Carbon/nitrogen	8.9	9.4	10.0	10.7	11.5	12.3	13.3	14.4	15.7	17.3	19.2

Eleven excreta-starch diets ranging from 0 to 50% starch (of DM, steps of 5%) were mixed keeping total dry mass at ~134 g and DM content at ~30%, resulting in a diet wet mass of ~440 g. Tap water was used to adjust dietary moisture levels with individual diets manually mixed using a wooden spatula in 1000 mL transparent polypropylene pots (product code 447692, Paardekooper packaging, Oud-Beijerland, the Netherlands) fitted with a mesh Bugdorm™ lid. Quadruplicates were made during four consecutive days with three inoculated with eggs and one replicate used to measure pH using a PHB-4 (INESA, Shanghai, China) with the probe inserted directly into the wet mass, after which the replicate was discarded.

Harvesting, sampling and storing of larvae and residues

During the 5-day larval growth period, temperature of the inoculated diets was measured daily using a probe thermometer (TFA Dostmann, Wertheim-Reicholzheim, Germany) inserted in the horizontal and vertical middle of each pot. On day 5, all pots were placed on melting ice to reduce larval and microbial metabolism. Representative portions (~50 g) of the residue (containing frass, leftover diet and exuviae) were cleared of larvae and frozen in polypropylene pots at -20 °C pending further processing. Larvae were collected from the remaining residues by floatation in 14% NaCl solution (Pisa *et al*. 2022), combined with the larvae from cleared residues and frozen at -20 °C. Before freeze-drying, larvae were thawed and any remaining debris was removed manually, after which larvae were randomly sampled and total larvae wet mass was weighed. Random sampling of larvae was conducted by laying a cardboard strip across the spread-out larvae mass and picking individuals directly adjacent to the strip until n = 30. The 30 larvae were weighed individually and returned to the total larvae mass, which was frozen again at -20 °C and freeze-dried. Before freeze-drying, residue portions were thawed and stirred, pH was measured by inserting the probe directly into the wet mass and samples (~5 g) were taken to determine ammonia content. Residues were frozen again at -20 °C and freeze-dried. All freeze-dried material was ground using a centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) using a 1 mm sieve at 12000 rpm and stored at -20 °C pending chemical analyses.

Chemical analyses

Ammonia was determined using a colorimetric method after deproteinisation with trichloroacetic acid based on Weatherburn (1967). Uric acid was determined in freeze-dried material by enzymatic-colorimetry using a commercial test kit (10694, Human GmbH, Wiesbaden, Germany). Freeze-dried material was analyzed for DM (ISO 6496 1999), starch (ISO 15914 2004), and total nitrogen (N) and carbon (C) by the Dumas method (ISO 16634-1 2008). All chemical analyses were performed in duplicate, except for DM (in simplo).

Calculations and statistical analyses

The average weight of a single housefly egg was determined as described by Pisa *et al*. (2022) and found to be 0.06 mg. This average was used to weigh portions of ~210 mg, corresponding to ~3500 eggs.

The number of larvae per pot was estimated by dividing total wet larvae weight by mean individual larva weight (n = 30) per pot. Mean individual larva weight and its uncertainty were derived from a generalized linear model (GLM) with a gamma distribution fitted to each pot separately. To calculate larval survival and incorporate the uncertainty derived from the individual larva weight GLM, a low and high larval count estimate was calculated per pot by dividing total wet larvae weight by the individual larva weight GLM lower and upper 95% confidence limits. Larval survival was then calculated by fitting a GLM with a binomial distribution and logit link to the ratio low, mean and high larvae count to number of eggs used per pot.

Bioconversion efficiency of DM (BE_{DM}) and N (BE_N) was calculated as the absolute amount (g) of DM and N found in the larval biomass divided by the absolute amount provided in the larvae diets, expressed as percentage. Loss of diet wet and dry mass was calculated as the absolute amount (g) per pot of diet wet mass weighed at the end of the larvae growing period divided by the absolute amount of diet wet mass at the start of the growing period. The fraction of starch recovered was calculated as the absolute amount of starch (g) per pot present in the residue, divided by the absolute amount of starch present in the diet, again expressed as a percentage.

Though relations between starch concentration and variables of interest were mostly non-linear, the low number of replicates combined with large between sample-variation favored simple linear methods for statistical inference, decreasing the chance on type 1 errors and over-fitting. Statistical inference was conducted by visual comparison of 95% confidence intervals (95% CI), with group means outside the confidence interval considered different and statistically significant at $p \leq 0.05$. Continuous fractions (BE, wet mass loss, dry mass loss, starch recovery) are bound by 0 and 100 (%) with the 95% CI based on a beta distribution (GLM with beta - logit link) using the 'betareg" package (Cribari-Neto and Zeileis 2010). Linear mixed models (LMM) were used for individual larva weight and total larvae wet weight using the package "lme4" (Bates *et al*. 2015). In the LMM and GLM used to generate the 95% CI, starch concentration and sterilization were used as an interaction-only categorical fixed effect with day (replicate) set as random intercept in LMM and as a fixed effect in beta GLM . All 95% CI were based on normal approximation (α =0.05, z = 1.96). All analyses were conducted in R version 3.6.1 (R Core Team 2019) running in R studio (R studio team 2019).

Results

Characteristics of fresh diets

After homogenization and sterilization, total N, total C and DM were not significantly different (within 1 SD) for UE and SE (Table 1). Unsterilized excreta contained more NH3 (~13%) and uric acid (~24%) than SE. The combined treatments had a major impact on the pH of the material with UE having a mean pH of 8.3 (SD: 0.08) whereas SE had a pH of 7.7 and a lower variability (SD: 0.02). Stirring in different amounts of gelatinized starch did not affect the starting pH of the experimental diets with all UE diets having a pH ~8.6 (SD: 0.05) while all SE diets had a pH of 7.6 (SD: 0.07).

Larval performance

Individual larva wet weight (Figure 1A) and its distribution (Figure S1, S2) differed between diets. For up to 15% starch, individual weights did not differ between SE and UE, showing a maximum of ~18 mg. After 15% starch addition, a decrease was observed to a minimum of ~5 mg individual weight, with a more pronounced initial drop in UE. After the maximum value, the SE diet generally yielded heavier larvae than the UE diet. For the UE diet, individual larval weights approximated a normal distribution (Figure S2) up to 10% starch. At higher starch concentrations, a tendency towards a right skewed distribution was observed. The SE diet yielded a distribution which was more flattened up to 10% starch, followed by at first left skewed distributions at 15 and 20% starch while showing right skew distributions at the highest starch concentrations. Total larvae wet weight (Figure 1B) peaked at 10% starch in the UE diet (39 g) and at 15% starch in the SE diet (43 g), decreasing to less than 5 and 2 g in the SE and UE diet above 35% starch, respectively. After its maximum at 15% starch, the SE diet always yielded more total larvae wet mass than UE. The estimated larval survival rate (Figure 1C) showed a more linear relation with starch concentration than individual and total larvae wet weight. Survival was highest and not different between the UE and SE diets up to 10% starch. At 15% and between 25 and 50% starch, survival was significantly higher in the SE diet. Larval C/N (Figure 1D) increased linearly by ~30% from 0% to 25% starch in the UE (5.2 to 7.5) and SE (5.5 to 7.2) diet. The C/N in the UE diet reached a maximum value at this point, after which it decreased, showing a large variation between replicates. The C/N in the SE diet continued to gradually increase to a maximum (~7.6) at 35 to 45% starch.

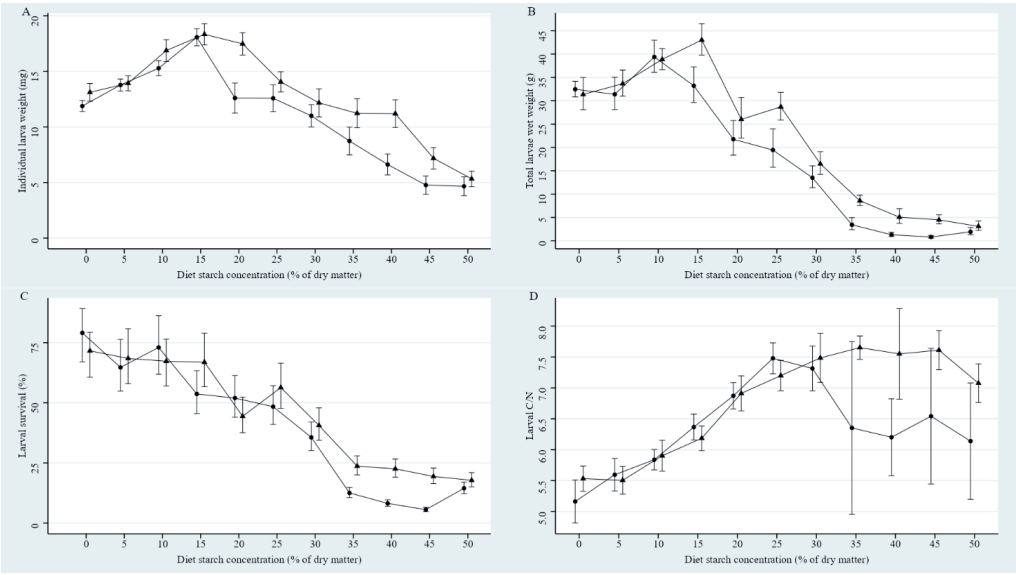


Figure 1. Housefly individual larva wet weight (A), total larvae wet weight (B), larval survival (C) and larval C/N (D) grown on unsterilized (●) or sterilized (▲) excreta diets with different starch content. Symbols with whiskers show means and 95% confidence intervals.

Diet mass loss, dietary starch recovery and residue C/N

Diet wet mass loss (Figure 2A) decreased in a linear manner in both diets, with the UE diet showing the highest loss (15%) at 5% starch, while the highest loss for the SE diet occurred at 5 to 15% starch. At the lowest starch concentrations, loss was higher for the UE diet but beyond 5% starch the SE di*et al*ways lost slightly more wet mass. Diet dry mass loss (Figure 2B) peaked at 5% starch in both diets and was again higher in the SE diet for most starch concentrations. The recovery of dietary starch (Figure 2C) showed a maximum of ~ 22% in the SE diet at 40% starch. Recovery was higher for the UE than the SE diet up to 25% starch but after this point recovery was considerably higher in the SE diet, with differences between diets never exceeding 10%. Residue C/N (Figure 2D) increased similarly in both diet residues up to 15% starch, compared to diet C/N. At starch concentrations higher than 15%, the residue C/N followed the diet C/N curve but always 10 to 20% lower.

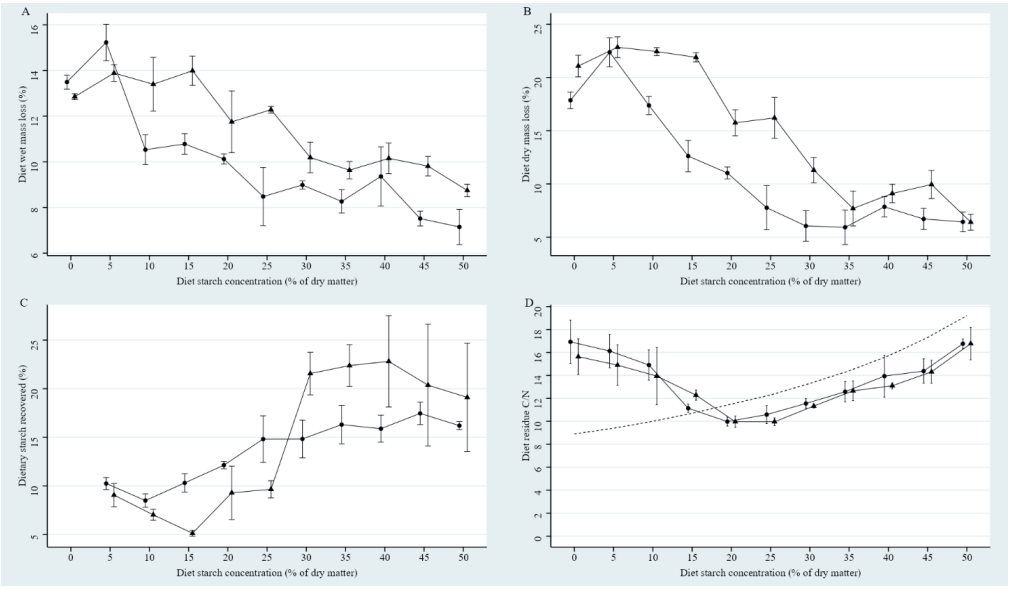


Figure 2. Wet mass loss (A), dry mass loss (B), starch recovery (C), and residue C/N ratio (D) in unsterilized (●) and sterilized (▲) excreta housefly larval diets with varying starch contents. The dashed line represents the C/N ratio of the fresh diet. Symbols with whiskers indicate means and 95% confidence intervals.

Dry matter and nitrogen bioconversion efficiency

Starch addition affected BE_{DM} and BE_N with the former (Figure 3A) increasing approximately 34% in the UE diet (4.2 at 0% starch to 6.4 at 10% starch) and approximately 46% in the SE diet (3.8 at 0% starch to 7.0 at 15% starch). After these maxima, BE_{DM} decreased linearly to values less than 1% at 40% starch. Bioconversion efficiency of nitrogen also increased significantly by low amounts of starch addition to the excreta (Figure 3B). The UE diet showed an increase of 38% (9.6 at 0% starch to 15.5 at 10% starch) while in the SE diet an increase of 50% (7.7 at 0% starch to 15.3 at 15% starch) was observed. As was the case with BE_{DM}, both diets decreased linearly after their maxima to ~15%

until the highest starch concentrations, with BE_{DM} of the SE diet being significantly higher over most of the starch concentration range.

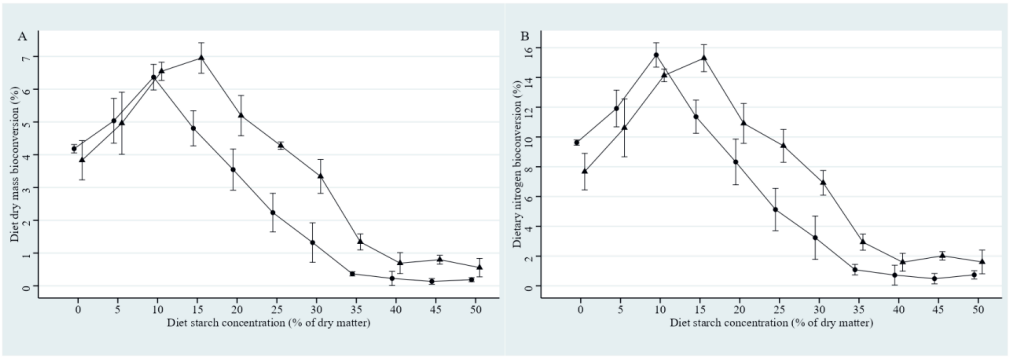


Figure 3. Dry matter bioconversion % (A) and nitrogen bioconversion % (B) of unsterilized (●) or sterilized (▲) excreta larval diets with different starch content. Symbols with whiskers show means and 95% confidence intervals.

Uric acid, ammonia and pH

At starch concentrations up to 10%, less than 10% of the uric acid theoretically present in the larval diet was recovered, with recovery significantly lower for the UE diet (Figure 4A). At 20% starch, the concentration of uric acid present in the residue (25 g/kg DM) was equal in both diets but considerably closer to the theoretical dietary concentration for the SE diet. For the latter diet, the maximum concentration of 25 g/kg DM occurred at 20% starch, whereas in the UE diet the maximum of 30 g/kg DM occurred at 25% starch. After their maxima, the uric acid concentrations declined in both diet, closely following the decline in the theoretical dietary concentrations. Ammonia concentrations in residues were higher than in the fresh diets up to 20% starch inclusion for both diets and decreased to values equal to those in the fresh diets over 25% starch inclusion (Figure 4B). In both diets, the decrease in NH3 concentration over the starch range was not different. Both UE and SE diet residues showed a lower pH with increasing starch concentration (Figure 4C). Up to 10% dietary starch content the pH of the residues did not differ between UE and SE. But at 15 to 25% starch, the pH showed a large difference between UE and SE diets, with SE showing a major decrease between 15 and 20% starch while in UE a similar decrease took place between 20 and 25% starch.

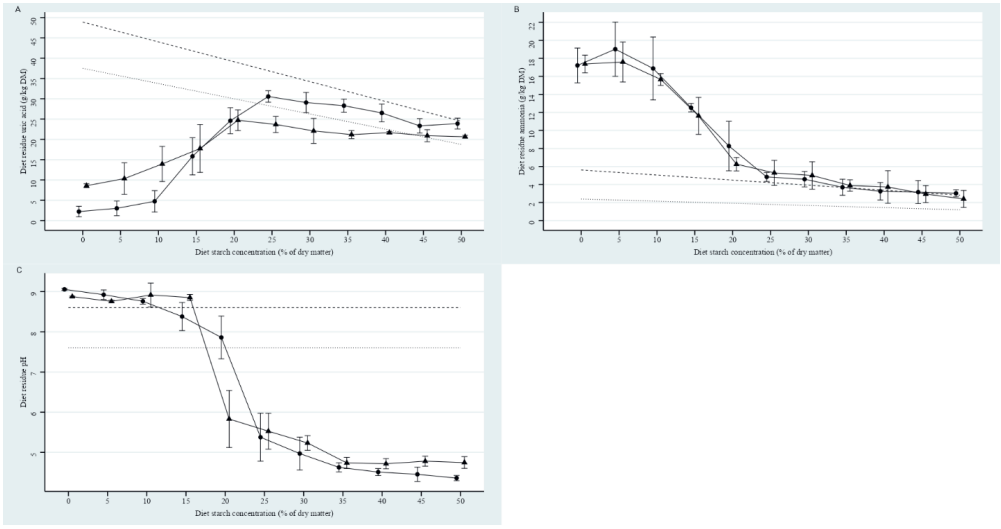


Figure 4. Diet residue uric acid content (A), diet residue ammonia content (B) and diet residue pH (C) of unsterilized (●) or sterilized (▲) excreta larval diets with different starch content. Symbols with whiskers show means and 95% confidence intervals. The dashed and dotted line represent the uric acid and ammonia concentrations and pH of fresh unsterilized and sterilized excreta diets, respectively. Symbols with whiskers show means and 95% confidence intervals.

Discussion

Addition of starch to the chicken excreta influenced growth, survival and BE of housefly larvae. Positive effects of starch addition on larval performance and BE were limited to the 5 to 15% starch range in both the UE and SE diet. Heat sterilisation of excreta tended to exert positive effects of starch on individual larva weight up to 20% and showed a lower decrease of larval performance and BE between 15 and 35% starch. Regardless of sterilisation, adding > 35% starch resulted in a decreased larval performance and BE below their values in starchless excreta.

From an energy from protein versus energy from carbohydrates perspective, shifting the balance towards easily digestible carbohydrates should increase individual growth, with added carbohydrate leading to heavier (larger) larvae that have used more of the available dietary protein (nitrogen) for body growth. Increased growth of individual larvae in relation to addition of 5 to 15% starch was found in this study, as well as an increase in total larval biomass, more larvae grew heavier with no significant effect on larval survival. This supports the hypothesis that larval growth and total larval biomass in excreta is limited by lack of easily digestible carbohydrates, with the optimal diet C/N ratio ranging from 9.4 to 10.7%. The increase in BEN with limited starch addition also points towards increased larval utilisation of dietary N. Larval C/N increased over the range of starch addition associated with heavier larvae and more total larvae biomass, potentially indicating that larvae increased in body fat.

The combination of higher survival and slower decrease of individual weight seems to explain the difference in peak total larval biomass between the diets. This combinatory effect is complicated by the distribution of individual larval weight at 10 and 15% starch in the SE diet (see Figures S1, S2).

At these starch concentrations, the distribution of individual larval weight showed a right shift, indicating that proportionally more heavier larvae were present compared to lower and higher starch concentrations. In this experiment, the decrease in larval weights was associated with a departure from the normal distribution towards at first left- (more heavy than light larvae) and right-skewed (more light than heavy larvae) distribution at higher starch concentrations. This relation was also observed, all be it less clear, by Pisa *et al*. (2022) and might be an indication of competition between larvae for nutrients or micro-conditions (aeration, moisture) in the substrate. Determining individual mass distribution might serve as a quality control measure in larva mass rearing, with non-normal distributions of individual traits, such as weight or length, indicating sub-optimal nutritional or other conditions.

Whereas the relation between starch addition and individual larvae mass and total larval biomass was curvilinear, the loss of wet mass was roughly linear. Though the wet mass loss over the starch concentration range peaked at 15% starch in the SE diet, concurring with the highest larval wet mass, this effect was small. The loss of wet mass was probably highly dependent on the experimental container, with the 1 L cylindrical pots having a small surface area relative to volume. The loss of dry matter did follow the curvilinear pattern seen for total larvae wet mass, indicating that the larval biomass did affect the change in dry mass. This effect could either be direct, through consumption by larvae, or indirect, by an effect of larvae on the microbiota present. The higher dry mass loss in sterilized excreta with its lower initial microbiota activity would indicate that the larvae are the main actor for dry mass loss. This is supported by the pattern of starch recovery from the diet residues where recovery in sterilised excreta was lower than that of unsterilised excreta up to 25% starch content, which matched the development of the total larval biomass. At concentrations > 25% starch, the total larval biomass was always less than that at 15% starch, starch recovery was always higher in sterilised excreta. Additional insight into the effect of larvae on substrate and microbiota is potentially seen in the pattern of uric acid remaining in the diet residues (Figure 4A). At starch concentrations in sterilised excreta > 20% and 25% in unsterilised excreta, uric acid recovery plateaued and then decreased, while at > 20-25% starch concentrations, more uric acid was recovered from unsterilized excreta diet residues. One explanation for this pattern is that the increasingly large larval biomass produced more uric acid that was not decomposed (to ammonia) by microbiota in the sterilised excreta diets. The recovery in sterilised excreta would then be higher initially because certain microbiota were inactivated. However, the amount of ammonia, the product of bacterial degradation of nitrogenous compounds such as uric acid, was found in both residue types to be remarkably similar. An alternative explanation is that increasing larval biomass reduced (certain) microbiota activity that decompose uric acid in the sterilised diets. Housefly larvae emit a broad range of antimicrobial compounds (review by Niode *et al*. 2022) and are associated with a specific bacterial fauna (De Jonge *et al*. 2020). A part of this fauna, e.g. the genera *Proteus* and *Morganella*, play a role by decreasing competition between larvae and other microbiota species, notably fungi (Lam *et al*. 2007). Because of the inability to separate larval and bacterial effects on the presence of uric acid in the residues in this study, the uric acid concentrations in residues could not support the hypothesis that adding starch to excreta decreased amino acid catabolism and larval nitrogen excretion. For future

experiments, it is, therefore, of interest to determine the composition of the microbiota in larval diets and take biochemical effects of specific microbiota involved into account when constructing hypotheses on larval nutrition.

A subjective though potentially useful indication for differences in microbiota are the generated odors with the different diets during the larval growth period. Unsterilised diets with up to 10% starch had a strong ammonia smell, while turning progressively "fruity" at higher starch concentrations. Sterilised diets with up to 10% starch also smelled of ammonia, but beyond 15 to 20% starch had a more "woody" (fresh wood chips, bread mold) odor. Volatile products of microbiota are used for species identification (Lemfack *et al*. 2018, Elmassry and Piechulla 2020) and developing methodology using artificial noses or gas chromatography might be very useful for process control in housefly larvae mass-rearing or for insect rearing in general.

Comparing bioconversion efficiencies (BE) found in the current study with the previous work by Pisa *et al*. (2022), who used sterilized and unsterilized excreta with 50% starch (of diet DM) and the same egg density (8 eggs / g wet diet), yields both similar and conflicting outcomes. For BE_{DM}, outcomes were somewhat lower for pure UE and SE, 3.5% and 2.6%, respectively, while for 50% starch in SE the outcome was much higher than in the current study, 3.1% versus 0.6%. Nitrogen-BE of starchless UE were highly similar, 9.6% and 9.3%, while BE_N of starchless SE was somewhat lower in the previous study, 7.6% versus 6.6%, respectively. A large difference between studies is seen for the 50% starch addition, with BE_N in the current study found to be much lower, 0.73% versus 4.0% in UE and 1.6% versus 9.9% in SE. This can be attributed to differences in diet mass and homogenization. Pisa *et al*. (2022) used a smaller amount (100 g) of unhomogenized excreta, in a ~7 cm deep layer in smaller pots with a relatively large opening, allowing for more aeration and a different drying profile of the diets. This stresses the fact that when designing bioconversion experiments, care must be taken to standardize how the diet is offered.

As far as known, there are no other publications of BE_N of chicken excreta by housefly larvae except for Pisa *et al*. (2022). For the black soldier fly, *Hermetia illucens*, another potential species for the bioconversion of animal manure, Zhang *et al*. (2023) found an BE_N value of 23.6% for pure unsterilized chicken manure, though it is unclear from their data if their chicken manure consisted of excreta or excreta with bedding. Several authors report BE_{DM} of pure unsterilized excreta or data that allow for its calculation but differences in scale, the type of excreta used and the stage in which larvae were harvested do not allow for valid comparisons. The BE_{DM} found in the current study are in the range of those found by Miller *et al*. (1974), 3% to 8% and 2% to 9% by Cai *et al*. (2017) who harvested fresh larvae using similar experimental set-ups.

For follow-up studies, two subjects are highly relevant. The first subject is the aforementioned relation between housefly larvae and microbiota. The second subject is the use of higher densities of larvae. The current density of 8 eggs / g wet diet, also used in Pisa *et al*. (2022), might be too low for optimal bioconversion. Higher larvae yields are reported for larval densities higher than 8 larvae / g wet diet (Hussein *et al*. 2017, Leyo *et al*. 2021) and housefly larvae, like other larvae from species with a similar feeding ecology, might need high densities to form aggregations that lead to improved

survival, nutrition and defense against pathogens (review by Fouche *et al*. 2021, Than *et al*. 2020). Using higher densities of larvae will generate insight on the role of larvae as actors in larval diets and give leads to further improve excreta bioconversion by housefly larvae.

Conclusions

This study support the hypothesis that housefly larval growth in chicken excreta is limited by available carbohydrates. Adding 5 to 15% starch to chicken excreta improved larval growth and larval biomass and subsequent bioconversion of dry matter and nitrogen. Doses > 20 to 25% showed a decrease in larval growth and bioconversion compared to starchless excreta. Heat sterilisation of chicken excreta showed a significant increase in larval growth and bioconversion in the 10 to 20% starch addition range, compared to unsterilised excreta. Uric acid decomposition, ammonia production, decrease of pH and decrease in diet residue C/N all showed a tipping point around 20 to 25% starch. The distributions of individual larva weight shifted from (flat) normal distributions at 0 to 15% starch to right skewed distributions at higher starch concentrations, potentially indicating increased competition for nutrients.

References

Bates, D., Mächler, M., Bolker, B., Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1-48.

Cai, Z., De-po, Y., Sheng-qing, W., Yong, W., Reaney, M. J. T., Zhi-min, Z., Long-ping, Z., Guo, S., Yi, N., Dong, Z., Hui-ran, N., Wen-zhe, Y. (2017). Conversion of poultry manure to biodiesel: A practical method of producing fatty acid methyl esters via housefly (*Musca domestica* L.) larval lipid. *Fuel*, 210, 463-471.

Čičková, H., Pastor, B., Kozánek, M., Martínez-Sánchez, A., Rojo, S., Takáč, P. (2012). Biodegradation of pig manure by the housefly, *Musca domestica*: A viable ecological strategy for pig manure management. *PLOS ONE*, 7, e32798.

Čičková, H., Newton, G. L., Lacy, R. C., Kozánek, M. (2015). The use of fly larvae for organic waste treatment. *Waste Management*, 35, 68-80.

Cribari-Neto, F., Zeileis, A. (2010). Beta regression in R. *Journal of Statistical Software*, 34(2), 1-24.

De Jonge, N., Michaelsen, T. Y., Ejbye-Ernst, R., Jensen, A., Elley Nielsen, M., Bahrndorff, S., Lund Nielsen, J. (2020). Housefly (*Musca domestica* L.) associated microbiota across different life stages. *Scientific Reports*, 10, 7842

Elmassry, M. M., Piechulla, B. (2020). Volatilomes of bacterial infections in humans. *Frontiers in Neuroscience*, 14, 257.

Fouché, Q., Hedouin, V., Charabidze, D. (2021). Effect of density and species preferences on collective choices: An experimental study on maggot aggregation behaviours. *The Journal of Experimental Biology*, 224, jeb233791.

Frank, K., Brückner, A., Hilpert, A., Heethoff, M., Blüthgen, N. (2017). Nutrient quality of vertebrate dung as a diet for dung beetles. *Scientific Reports*, 7, 12141.

Gadzama, I. U., Ndudim, R. K. (2019). Nutritional composition of housefly larvae meal: A sustainable protein source for animal production - a review. *Acta Scientific Agriculture*, 3, 74-77.

Goh, Y. J., Klaenhammer, T. R. (2014). Insights into glycogen metabolism in *Lactobacillus acidophilus*: Impact on carbohydrate metabolism, stress tolerance and gut retention. *Microbial Cell Factories*, 13, 94.

Hirashima, A., Oyama, K., Ueno, R., Ishaaya, I., Eto, M. (1989). Carbohydrate enzymes in house fly *Musca domestica* larvae: Biochemical properties and toxicological significance. *Journal of the Faculty of Agriculture, Kyushu University*, 34, 37-42.

Hogsette, J. A. (1992). New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. *Journal of Economic Entomology*, 85(6), 2291-2294.

Holter, P., Scholz, C. H. (2007). What do dung beetles eat? *Ecological Entomology*, 32(6), 690-697.

van Huis, A. (2019). Manure and flies: Biodegradation and/or bioconversion? *Journal of Insects as Food and Feed*, 5(2), 55-58.

Hussein, M., Pillai, V. V., Goddard, J. M., Park, H. G., Kothapalli, K. S., Ross, D. A., Ketterings, Q. M., Brenna, J. T., Milstein, M. B., Marquis, H., Johnson, P. A., Nyrop, J. P., Selvaraj, V. (2017). Sustainable production of housefly (*Musca domestica*) larvae as a protein-rich feed ingredient by utilizing cattle manure. *PLoS ONE*, 12(2), e0171708.

ISO 6496 (1999). Animal feeding stuffs – Determination of moisture and other volatile matter content. International Organization for Standardization, Geneva, Switzerland.

ISO 15914 (2004). Animal feeding stuffs - Determination of total starch content. International Organization for Standardization, Geneva. Switzerland.

ISO 16634-1 (2008). Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content - Part 1: Oilseeds and animal feeding stuffs. International Organization for Standardization, Geneva, Switzerland.

Khan, H. A., Shad, S. A., Akram, W. (2012). Effect of livestock manures on the fitness of house fly, Musca domestica L. (Diptera: Muscidae). Parasitology Research, 111(3), 1165-1171.

Kurbanoglu, E., Algur, O. (2002). Single-cell protein production from ram horn hydrolysate by bacteria. *Bioresource Technology*, 85(2), 125-129.

Lalander, C., Vinnerås, B. (2022). Actions needed before insects can contribute to a real closed-loop circular economy in the EU. *Journal of Insects as Food and Feed*, 8(4), 337-342.

Lam, K., Babor, D., Duthie, B., Babor, E. M., Moore, M., Gries, G. (2007). Proliferating bacterial symbionts on house fly eggs affect oviposition behavior of adult flies. *Animal Behaviour*, 74, 81-92.

Lemfack, M. C., Gohlke, B. O., Toguem, S. M. T., Preissner, S., Piechulla, B., Preissner, R. (2018). mVOC 2.0: A database of microbial volatiles. *Nucleic Acids Research*, 46, 1261-1265.

Leyo, I. H., Ousmane, M. Z., Noël, G., Francis, F., Megido, C. R. (2021). Breeding enhancement of *Musca domestica* L. 1758: Egg load as a measure of optimal larval density. *Insects*, 12(11), 956.

Miller, B. F., Teotia, J. S., Thatcher, T. O. (1974). Digestion of poultry manure by *Musca domestica*. *British Poultry Science*, 15(2), 231-234.

Miranda, C. D., Cammack, J. A., Tomberlin, J. K. (2019). Life-history traits of house fly, *Musca domestica* L. (Diptera: Muscidae), reared on three manure types. *Journal of Insects as Food and Feed*, 6, 81-90.

Murakami, K., Hara, M., Kondo, T., Hashimoto, Y. (2011). Increased total nitrogen content of poultry manure by decreasing water content through composting processes. *Soil Science and Plant Nutrition*, 57(5), 705-709.

Nahm, K. (2003). Evaluation of the nitrogen content in poultry manure. *World's Poultry Science Journal*, 59(1), 77-88.

Niode, N. J., Mahono, C. K., Lolong, F. M., Matheos, M. P., Kepel, B. J., Tallei, T. E. (2022). A review of the antimicrobial potential of *Musca domestica* as a natural approach with promising prospects to countermeasure antibiotic resistance. *Veterinary Medicine International*, Article ID 9346791.

Pisa, L. W., Oonincx, D. G. A. B., Bosch, G., Hendriks, W. H. (2022). Bioconversion of fresh chicken excreta by housefly larvae (*Musca domestica* L.) in relation to excreta sterilisation and carbohydrate addition. *Journal of Insects as Food and Feed*, 8(10), 1107-1116.

Pluske, J., Montagne, L., Cavaney, F., Mullan, B., Pethick, D., Hampson, D. (2007). Feeding different types of cooked white rice to piglets after weaning influences starch digestion, digesta and fermentation characteristics and the faecal shedding of β-haemolytic *Escherichia coli*. *British Journal of Nutrition*, 97(2), 298-306.

R Core Team. (2019). *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria.

Ritala, A., Häkkinen, S. T., Toivari, M., Wiebe, M. G. (2017). Single cell protein - state-of-the-art, industrial landscape and patents 2001-2016. Frontiers in Microbiology, 8, 2009.

RStudio Team. (2019). *RStudio: Integrated development for R.* RStudio, Inc., Boston, MA.

Sánchez-Muros, M. J., Barroso, F. G., Manzano-Agugliaro, F. (2014). Insect meal as renewable source of food for animal feeding: A review. *Journal of Cleaner Production*, 65, 16-27.

Shah, R. M., Azhar, F., Shad, S. A., Walker, W. B., Azeem, M., Binyameen, M. (2016). Effects of different animal manures on attraction and reproductive behaviors of common house fly, *Musca domestica* L. *Parasitology Research*, 115, 3585-3598.

Sinha, M. (1975). Amylase activity in the midgut of *Sarcophaga ruficornis* and *Musca domestica*. *Entomologia Experimentalis et Applicata*, 18(3), 290-296.

Terra, W. R., Jordão, B. P. (1989). Final digestion of starch in *Musca domestica* larvae: Distribution and properties of midgut α-D-glucosidases and glucoamylase. *Insect Biochemistry*, 19(3), 285-292.

Than, A. T., Ponton, F., Morimoto, J. (2020). Integrative developmental ecology: A review of density-dependent effects on life-history traits and host-microbe interactions in non-social holometabolous insects. *Evolutionary Ecology*, 34, 659-680.

Wang, L., Liu, Q., Hu, J., Asenso, J., Wise, M. J., Wu, X., Ma, C., Chen, X., Yang, J., Tang, D. (2018). Structure and evolution of glycogen branching enzyme N-termini from bacteria. *Frontiers in Microbiology*, 9, 3354.

Weatherburn, M. W. (1967). Phenol hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, 39, 971-974.

Weihrauch, D., O'Donnell, M. J. (2021). Mechanisms of nitrogen excretion in insects. *Current Opinion in Insect Science*, 4, 25-30.

Weurding, R. E., Veldman, A., Veen, W. A. G., van der Aar, P. J., Verstegen, M. W. A. (2001). Starch digestion rate in the small intestine of broiler chickens differs among feedstuffs. *Journal of Nutrition*, 131(9), 2329-2335.

Zhang, H., Zhang, X., Chen, M., Deng, X., Pei, Y., Zhang, J., Chen, H., Yang, S. (2023). Biochar can improve absorption of nitrogen in chicken manure by black soldier fly. *Life*, 13(4), 938.

Supplementary material

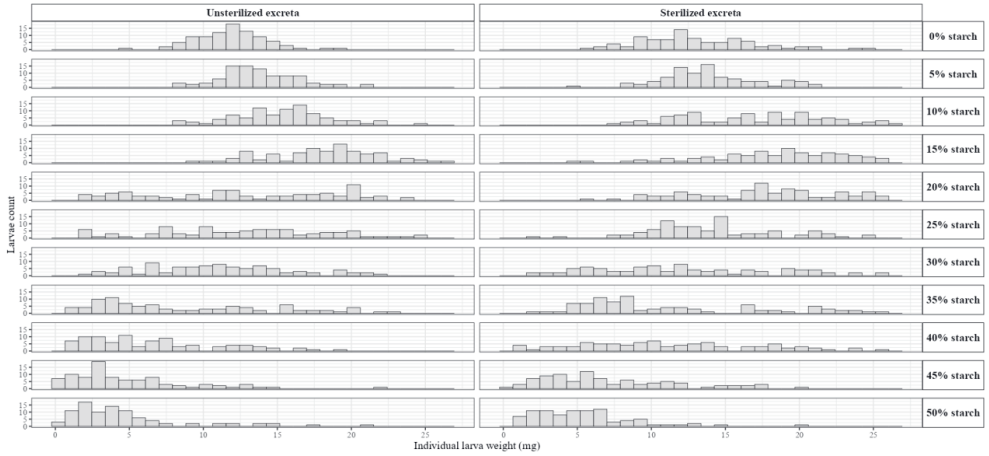


Figure S1. Histograms of individual housefly larva wet mass samples (n=30, N=3) in relation to excreta sterilization and diet starch content.

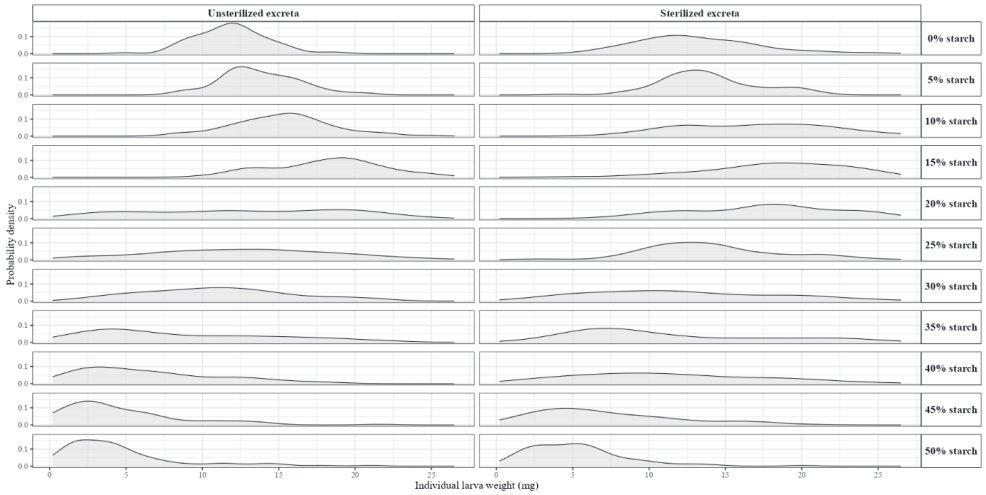


Figure S2. Fitted probability density of individual housefly larva wet mass samples (n=30, N=3) in relation to excreta sterilization and diet starch content

.

Chapter 4

Timing of digestible carbohydrate addition to fresh chicken excreta affects housefly (*Musca domestica*) larval growth and bioconversion

L.W. Pisa, G. Bosch, W.H. Hendriks, D.G.A.B. Oonincx

Submitted to Journal of Insects as Food and Feed

Abstract

Mass-rearing of housefly larvae (*Musca domestica* L.) on livestock manure can contribute to sustainable feed production and waste reduction. Fresh chicken excreta contain little digestible carbohydrate and supplementation may enhance larval biomass and nitrogen retention, but larval use of carbohydrates may depend on developmental stage. This study tested how the type (glucose, starch, cellulose, fresh excreta as control) and timing (0 or 72 h) of supplementation affected larval growth, bioconversion and residue characteristics. Glucose and starch substantially improved larval performance compared with fresh manure and cellulose. Late (72 h) supplementation, was most effective, with late glucose increasing individual larval mass by 12%, total biomass by 33% and survival by 19%. Late starch increased total biomass by 30% and survival by 24%, while effects on individual mass were small (2% increase). Dry matter bioconversion efficiency increased by 27% with late glucose and 20% with late starch, whereas late excreta addition caused an 18% decline. Nitrogen bioconversion rose by 36% with late glucose and 37% with late starch. Residue ammonia increased with all supplementation but less for 72 h additions, especially for starch. Residue pH increased with all supplementation, excreta and cellulose showed the largest early-late increases, early and late glucose showed the smallest change and late starch produced the overall lowest pH. The results support the hypothesis that supplementation of fresh chicken manure with starch later in the larval development period results in larger biomass, improved survival and higher bioconversion.

Introduction

The housefly (*Musca domestica*) is gaining industrial interest for the bioconversion of organic waste. Its larvae are able to utilize animal feces and other waste streams characterized by a high bacterial load and provide an end product with a high protein and fat content (Gadzama and Ndudim 2019). Larvae and derived larvae meal are suitable as feed ingredient and can substitute the fish meal and soybean meal currently used in aquaculture and livestock diets (Hwangbo *et al*. 2009, Elahi *et al*. 2020, Saleh 2020). Housefly larvae have a relatively fast growth cycle, a low susceptibility to pathogens and decrease noxious compounds in manure residues (Cickova *et al*. 2012, Zhang *et al*. 2012). Globally, an estimated 329 million tonnes of chicken manure were produced by broilers and layers in 2020, with demand for chicken meat and eggs still increasing (FAO 2023). Mass-rearing of housefly larvae could play a significant role in upcycling nitrogen from chicken manure to animal feed. In the last decades, successful pilot projects investigated the scale up of larvae rearing but certain aspects of mass rearing such as increasing larval survival and optimalization of waste for larval nutrition remain a challenge (Pastor *et al*. 2015, Van Huis *et al*. 2020).Understanding the nutrient metabolism of larvae is important to optimize the conversion of excreta into larval biomass. Larvae require essential amino acids from proteins and energy for maintenance and growth. The available studies yield different insights on carbohydrate utilization by housefly larvae. Larvae grown on sterilized casein-based diets did not require digestible carbohydrates for their development (Brookes and Fraenkel 1958). The same was observed by Chang and Wang (1958) who also used sterilized semi-synthetic media. However, different amylases were found in the midgut of third-instar larvae, indicating that larvae can digest starch-like carbohydrates in their diet (Sinha 1975, Terra *et al*. 1988, Lemos and Terra 1992).

Although amylases can be present in the larval (mid)gut, their role in manure digestion is questionable. Fresh feces and excreta are nearly devoid of digestible carbohydrates, as ileal digestibility and bacterial utilization are high (Weurding *et al*. 2001, Rehman *et al*. 2009). Yet, housefly larvae can use carbohydrates added to chicken excreta in situ. Starch addition to fresh and sterilized excreta increased individual and total larval weight and improved nitrogen bioconversion efficiency of excreta, with higher doses of starch showing stronger effects in sterilized excreta, and all doses decreasing larval survival (Pisa *et al*. 2022, 2024).

In contrast to the absence of digestible carbohydrates, fresh chicken excreta contains up to 30% crude protein, largely due to the die-off and lysis of gut bacteria after defecation, which releases protein and other intracellular contents (Smith and Wheeler 1979, Nahm 2005). This microbial protein has a well-balanced amino acid profile and high digestibility (Glencross *et al*. 2020, Ayodele *et al*. 2024). Pisa *et al*. (2022) speculated that young larvae predominantly depend on this abundant, soluble protein for growth and energy, whereas older larvae may be more nutritionally flexible. Older larvae can ingest intact bacteria that colonize the substrate over time, with several species such as *Escherichia coli* and *Lactobacillus* sp. potentially containing digestible carbohydrates in the form of glycogen that can be digested by housefly amylases (Kumari *et al*. 2012, Sekar *et al*. 2020, Esteban-Torres *et al*. 2023).

This combination of temporal nutrient availability and ontogenetic differences suggest that the timing of nutrient suppletion could influence larval growth and energy use. In this study, the timing of easily digestible carbohydrates addition on larval performance is investigated to test the hypothesis that older (second-/third-instar) compared to younger (first-instar) larvae can better utilize easily digestible carbohydrates added to fresh chicken excreta.

Materials and methods

Experimental design

A 4×2 factorial design was used with four substrates and two addition time points. Experimental substrates were pure chicken excreta, or excreta with either gelatinized corn starch, glucose or cellulose. Gelatinized corn starch, which can be utilized by larvae, was of main interest due to time-dependent occurrence of larval amylases. Glucose was used as a positive control as larvae are able to use this carbohydrate directly regardless of larval instar, the effect on larval performance and bioconversion should be similar to that to that of gelatinized corn starch if larval performance on excreta is limited only by lack of easily digestible carbohydrate. Cellulose was used as a negative control, as it is undigestible for larvae and controls for the effect of stirring an exogenous material into the larval substrate, potentially disturbing the larvae. The time of addition was either during the substrate mixing before inoculation with eggs (early) or 72 h after inoculation (late). The 72 h-point was chosen based on the findings of Wang *et al*. (2018), who found the development from egg to third instar larvae to be approximately 60 h, so most if not all larvae would be third instar at 72 h. Larvae were harvested from all substrates at 120 h post-inoculation. Six replicates were made of all eight substrate-time combinations, with three replicates inoculated with eggs on two consecutive days using pooled eggs from different cages of flies (n = 24, N = 48).

Housefly egg production

A housefly culture was established at Wageningen University & Research in September 2021 using larvae of the Dutch GK strain provided by Groningen University, the Netherlands. Adult flies were kept in mesh Bugdorm™ cages of 47.5×47.5×93 cm (MegaView Science Co., Taichung, Taiwan) with approximately 3000 individuals per cage in a climate room (27 °C., 60% RH, 16:8 light-darkness). Flies were provided ad libitum with a thin layer of milk-based baby formula (0 to 6 months standard, Albert Heijn home brand, Zaandam, the Netherlands) and sucrose cubes (Albert Heijn home brand, Zaandam, the Netherlands) on disposable plastic plates. Tap water was provided in polypropylene pots with cotton wicks inserted through the lids. Fresh food and water were provided every ~48 h. Larvae were reared in the same climate room in 1500 ml black food grade polypropylene trays without lids (RE180 series, Disposable Discounter, Groningen, the Netherlands). Larval substrate was composed of one part dry extruded dog kibble (Albert Heijn adult lamb and rice, Partner in Pet Food, Doetinchem, the Netherlands), soaked in two parts water and covered with 1.5 parts (by weight) of dry wheat bran (Meneba, Rotterdam, the Netherlands). The population was sustained by allowing flies to oviposit on fresh substrate and partitioning the resulting substrate with young larvae after 48 h into new substrate trays at 400-500 larvae to ~300 g fresh substrate. After pupation, trays

with pupae were placed in clean cages for eclosion. Housefly eggs were collected and the average weight of a single housefly egg was determined as described by Pisa *et al*. (2022) and found to be 0.065 mg. This average was used to weigh portions of ~254 mg, corresponding to ~3900 eggs, approximately 8 eggs per g wet substrate.

Experimental substrates

Fresh (<24 h old) excreta of layer hens fed a commercial wheat-soybean based feed were obtained from Rondeel farm (Barneveld, the Netherlands) and homogenized using a FGC 10-2 bowl cutter (Feuma Gastromaschinen GmbH, Gößnitz Germany). During homogenization, feathers and egg shells were manually removed and samples taken for rapid moisture content determination and chemical analysis. Moisture content of excreta, gelatinized corn starch (Research Diet Services, Wijk bij Duurstede, the Netherlands), glucose (Sidroga Pharma, Bad Ems, Germany) and cellulose (Arbocel, BWW40, Rettenmaier & Söhne, Rosenberg, Germany) was determined in triplicate (5 g material) with a MB 120 moisture analyzer (OHAUS Europe GmbH, Nänikon, Switzerland) before mixing the substrates. Dry matter (DM) content of glucose, starch and cellulose were found to be 983.0 ± 8.1, 898.4 ± 4.3 and 951.0 ± 8.4 (mean ± SD) g/kg, respectively.

Starch, glucose or cellulose was added as 10% of the substrate total DM, taking their moisture content into account. Carbohydrates were added either at the start or at 72 h of the experiment, resulting in early glucose (EG), starch (ES) and cellulose (EC) and late glucose (LG), starch (LS) and cellulose (LC) treatments. Pure excreta substrates consisted of only excreta, with all excreta added at the start of the larval growth period (early excreta, EE) or a portion at 72 h as 10% of total DM (late excreta, LE). Substrates were standardized to total dry mass and moisture content, with every diet containing ~153 g dry mass at ~30% DM, resulting in a diet wet mass of ~491 g per tray. Composition of chicken excreta and substrate composition are provided in Table 1 and 2.

Table 1. Analyzed composition (g/kg dry matter, average ± SD, n = 3) of chicken excreta

Item	Excreta
Dry matter (% as is)[1]	499.3 ± 3.7
Starch	0.5 ± 0.1
Uric acid	49.7 ± 3.7
Total C	380.2 ± 5.3
Total N	41.1 ± 1.1

Experimental procedure

Excreta were thawed overnight to 5 °C and adjusted for moisture content using tap water (Table 2) while constructing the substrates. Substrates were placed in 1.5 L (180×133×93 mm) transparent food grade polypropylene trays (RE180 series, Disposable Discounter, Groningen, the Netherlands) covered with pieces of fly screen to prevent oviposition by escaped flies. The EE substrates were made by adding the excreta all at once to a tray, gently stirring the material and forming a uniform layer of approximately 6 cm depth. The EG, ES and EC substrates were made by adding the excreta

to the tray, forming a uniform layer, followed by gently stirring in the powdered addition with a large inox fork and addition of (tap) water to adjust for the moisture content of the dry addition. Substrate pH was measured with a PHB-4 pH meter (INESA, Shanghai, China) directly after mixing by inserting the probe into the wet mass. The addition of material at 72 h to the "late substrates", LE, LS, LG and LC, was done by gently moving half of the excreta across the length of the tray to the side, sprinkling in half of the dry addition, adding half of the moisture correction water drip wise over the powdered addition and covering this up with the moved excreta. This was repeated for the other half of the tray, creating an uniform moistened layer of the addition in the excreta mass. The EE substrates were also stirred at 72 h to equalize mechanical effects on larvae. Inoculated trays were set in the dark (shelves surrounded by plastic sheeting) under the same conditions as used for the housefly culture.

Table 2. Ingredient composition and formulated chemical compositions of chicken excreta substrates, with addition of excreta, glucose, starch or cellulose at the start (early) or after 72 h (late).

Item	Substrate							
	Excreta		Starch		Glucose		Cellulose	
	Early	Late	Early	Late	Early	Late	Early	Late
Ingredient composition (g)								
Excreta 0 h	300	270	270	270	270	270	270	270
Excreta 72 h	0	30	0	0	0	0	0	0
Starch 0 h	0	0	17	0	0	0	0	0
Starch 72 h	0	0	0	17	0	0	0	0
Glucose 0 h	0	0	0	0	15.5	0	0	0
Glucose 72 h	0	0	0	0	0	15.5	0	0
Cellulose 0 h	0	0	0	0	0	0	16	0
Cellulose 72 h	0	0	0	0	0	0	0	16
Water 0 h	190	170	203	170	204	170	204	170
Water 72 h	0	19	0	33	0	34	0	34
Chemical composition (g/kg dry matter)								
Dry matter (g/kg as is)[1]	300	300	300	300	300	300	300	300
Starch	0	0	100	100	0	0	0	0
Glucose	0	0	0	0	100	100	0	0
Cellulose	0	0	0	0	0	0	100	100
Uric acid[2]	49.7	49.7	44.7	44.7	44.7	44.7	44.7	44.7
Ammonia[2]	2.1	1.9	1.9	1.9	1.9	1.9	1.9	1.9
pH[2]	7.85	7.84	7.83	7.83	7.81	7.83	7.84	7.84
C/N[1]	9.3	9.3	10.4	10.4	10.3	10.3	10.4	10.4

[1] Based on total material going in and estimated dry matter content. [2] Based on time = 0 h

Harvesting and processing of larvae and residues

During the 120 h (5 day) larval growth period, temperature of the substrates was measured daily between 9:00 and 11:00 using a probe thermometer (TFA Dostmann, Wertheim-Reicholzheim,

Germany) inserted in the horizontal and vertical middle of each pot. On day 5, portions (~50 g) of the substrate residue were cleared of larvae and frozen in polypropylene pots at -20 °C pending further processing. Larvae were collected from the remaining residues by floatation in 14% NaCl solution (Pisa *et al*. 2022), combined with the larvae from cleared residues and frozen at -20 °C. Before freeze-drying, larvae were thawed and any remaining debris was removed manually, after which larvae were randomly sampled and total larval biomass mass was weighed as described in Pisa *et al*. (2022). Before freeze-drying, the residue portions were thawed, gently stirred with an inox fork and pH was measured by inserting the probe directly into the wet mass and samples (~5 g) were taken to determine ammonia content.

Residues were frozen again at -20 °C and freeze-dried. All freeze-dried material was ground using a centrifugal mill (ZM 200, Retsch GmbH, Haan,Germany) using a 1 mm screen at 12000 rpm and stored in polyethylene pots at room temperature pending chemical analyses.

Chemical analyses

Uric acid was determined in freeze-dried material by enzymatic-colorimetry using a commercial test kit (10694, Human GmbH, Wiesbaden, Germany). Ammonia was determined using a colorimetric method based on Weatherburn (1967). DM (ISO 6496, 1999), starch (ISO 15914, 2004) and total nitrogen (N) and carbon (C) by the Dumas method (ISO 16634-1, 2008) were analyzed in freeze dried material. All chemical analyses were performed in duplo, except for DM (in simplo).

Calculations and statistical analyses

The number of larvae per pot was estimated by dividing total wet larvae weight by mean individual larva weight (n=30) per pot. Mean individual larva weight and its uncertainty were derived from a generalized linear model (GLM) with a gamma distribution fitted to each pot separately. A low and high larval count estimate was calculated per pot by dividing total wet larvae weight by the individual larva weight GLM lower and upper 95% confidence limits to calculate larval survival and incorporate the uncertainty derived from the individual larva weight GLM. Larval survival was calculated by fitting a GLM with a binomial distribution and logit link for the ratio low, mean and high larvae count to number of eggs used per pot, using interaction of addition and addition time as fixed effect and inoculation day as random intercept and slope, assuming 100% eclosion.

Bioconversion efficiency of DM (BE_{DM}) and N (BE_N) were calculated as the DM and N weight (g) in the larval biomass divided by the DM and N weight in the substrates, expressed as a percentage. Substrate wet and dry mass losses were calculated as the substrate wet or dry mass (g) per pot at the end of the larvae growing period divided by the wet or dry mass per pot at the start. The fraction of starch recovered was calculated as the absolute amount of starch (g) per pot present in the residue, divided by the absolute amount of starch present in the substrate, expressed as a percentage. Nitrogen emission was calculated as the absolute N (g) present in the substrate at start, minus the absolute amount of N recovered from the substrate residue and the absolute amount of N present in the larval biomass larvae, divided by the absolute amount of N present in the substrate at start, expressed as a percentage.

The area under the temperature-time curve (AUC) was calculated for each replicate using the trapezoidal numerical integration function from the pracma package (Borchers 2022) in R. Temperature values were integrated over time (0-120 h) to obtain a single measure of cumulative heat production per treatment. The resulting AUCs were analyzed using a two-way ANOVA with addition and addition time as fixed factors, followed by Tukey-adjusted pairwise comparisons.

Individual larvae weight, total larvae biomass, pH and larval C/N were compared using a linear mixed model that included the interaction of addition and addition time as fixed effect and inoculation day as random intercept and slope. Models were run using R package lme4 (Bates *et al*. 2015). Continuous fractions (BE_{DM}, BE_N, mass losses, N emission, uric acid content, NH_3 content) were compared using a beta distribution (GLM with beta - logit link) using the R package "betareg" (Cribari-Neto and Zeileis 2010). The interaction between addition and addition time was set as fixed effect and as "betareg" cannot include random effects, inoculation day was included as a fixed effect to separate error variances. For pairwise comparisons, the Tukey HSD method was applied. Residuals of all models were visually inspected for patterns and normality. To investigate associations between quantitative and qualitative variables, a factor analysis for mixed data was conducted using the R package " FactoMineR" (Lê *et al*. 2008). All analyses were conducted in R version 4.4.1 (R Core Team 2024).

Results

Addition of starch, glucose or cellulose to chicken excreta at 0 h did not lead to discernible changes in substrate viscosity during the stirring process, nor did it affect start pH. Temperature profiles differed between early and late additions (Figure S1). For excreta, EE and LE peaked at 48 h (39.5 °C and 39.1 °C), with a faster decline in LE. For glucose, EG and LG also peaked at 48 h (38.0 °C and 36.8 °C), but the LG peak persisted to 72 h (36.5 °C) and declined more slowly. For starch, ES and LS peaked at 48 h (36.8 °C and 38.6 °C), with LS maintaining a secondary peak at 72 h (37.6 °C) before a slower decline to 96 h. For cellulose, EC and LC peaked at 48 h (36.8 °C and 36.5 °C), with LC declining faster after 72 h. The integrated temperature response (AUC) differed significantly among substrates ($p < 0.001$) and showed a strong interaction between substrate and addition timing ($p < 0.001$). Post hoc comparisons confirmed that timing effects were substrate-specific. In starch, late addition increased total heat production by about 5% (≈ +1.6 °C average over 120 h) compared to early addition ($p < 0.001$), whereas in cellulose, late addition reduced it by a similar ~5% (≈ -1.7 °C average over 120 h, $p < 0.001$). In contrast, excreta and glucose showed smaller, non-significant timing-related differences (≤ 2%, $p > 0.2$).

Larval growth and survival

Individual larval weight, total larval weight and larval survival showed a similar pattern in relation to timed additions (Table 3). Compared to 0 h, addition of excreta at 72 h decreased individual weight (EE: 10.9 mg, LE: 10.6 mg, $p = 0.076$), total larval weight (EE: 28.2 g, LE: 24.7 g, $p = 0.044$) and survival (EE: 60.6%, LE: 55.1%, $p = 0.063$). Addition of glucose at 72 h increased individual weight

(EG: 12.3 mg, LG: 13.8 mg, $p < 0.001$), total larval weight (EG: 36.1 g, LG: 48.1 g, $p < 0.001$) and survival (EG: 69.0%, LG: 82.0%, $p < 0.001$).

Addition of starch at 72 h slightly increased individual weight (ES: 11.4 mg, LS: 11.6 mg, $p = 0.058$) and substantially increased total larval weight (ES: 28.3 g, LS: 36.7 g, $p < 0.001$) as well as survival (ES: 58.6%, LS: 72.7%, $p < 0.001$). Addition of cellulose at 72 h did not affect individual weight, total larval weight, nor survival (all $p > 0.961$). Overall, addition of glucose at 0 h increased individual and total larval weights compared to 0 h and 72 h excreta, 0 h starch, and 0 h and 72 h cellulose (all $p < 0.05$). Effects of glucose at 0 h on individual and total weight and survival were comparable to those of starch at 72 h ($p = 0.671$, 1.000, and 0.180, respectively).

Distribution of individual weights was generally flat-normal, with cellulose additions showing the most normality and late additions of excreta, glucose, and starch exhibiting slight right skew (Figure S2). Larval DM ranged from 163 g/kg for LC to 220 g/kg for ES (Table 3). Additions at 72 h consistently but not significantly reduced larval dry matter compared to 0 h, with excreta (EE: 205.6, LE: 193.4 g/kg, $p = 0.863$), glucose (EG: 194.6, LG: 183.7 g/kg, $p = 0.918$), starch (ES: 219.6, LS: 204.7 g/kg, $p = 0.097$) and cellulose (EC: 175.0, LC: 162.9 g/kg, $p = 0.869$). The C/N ratio of dried larvae (Table 3) decreased with late addition of excreta (EE: 5.44, LE: 5.15, $p = 0.040$) and cellulose (EC: 5.38, LC: 5.07, $p = 0.040$), remained unchanged for glucose (EG: 5.55, LG: 5.48, $p = 0.990$) and increased slightly for starch (ES: 5.51, LS: 5.58, $p = 0.970$).

Table 3. Individual larval wet weight, total larval wet weight, survival, dry matter content and C/N of housefly larvae grown on chicken excreta with addition of excreta, glucose, starch or cellulose at the start (early) or after 72 h (late).

Parameter	Substrate							
	Excreta		Glucose		Starch		Cellulose	
	Early	Late	Early	Late	Early	Late	Early	Late
Individual wet weight (mg)	10.9 ^{a} ± 0.3	10.6 ^{a} ± 0.3	12.3 ^{b} ± 0.3	13.8 ^{c} ± 0.4	11.4 ^{a} ± 0.4	11.9 ^{b} ± 0.3	10.8 ^{a} ± 0.3	10.7 ^{a} ± 0.3
Total wet weight (g)	28.2 ^{a} ± 3.1	24.7 ^{b} ± 2.5	36.1 ^{c} ± 2.1	48.1 ^{d} ± 2.4	28.3 ^{a} ± 2.2	36.7 ^{c} ± 3.1	28.5 ^{a} ± 2.2	28.4 ^{a} ± 2.3
Survival (%)	60.6 ^{a} ± 5.3	55.1 ^{a} ± 5.4	69.0 ^{b} ± 5.3	82.0 ^{c} ± 5.3	58.6 ^{a} ± 5.3	72.7 ^{b} ± 5.3	61.9 ^{a} ± 5.3	62.2 ^{a} ± 5.3
Dry matter content (g/kg)	205.6 ^{a} ± 12.7	193.4 ^{a} ± 12.7	194.6 ^{a} ± 12.7	183.7 ^{a} ± 12.7	219.6 ^{a} ± 12.7	204.7 ^{a} ± 12.7	175.0 ± 12.7	162.9 ^{b} ± 12.7
C/N	5.4 ^{a} ± 0.2	5.1 ^{b} ± 0.2	5.5 ^{a} ± 0.2	5.5 ^{a} ± 0.1	5.5 ^{a} ± 0.1	5.6 ^{a} ± 0.2	5.4 ^{a} ± 0.1	5.1 ^{b} ± 0.2

Data presented as mean ± margin of error and based on n = 6. Means with different superscript letters within row differ significantly (Tukey-adjusted, $p \leq 0.05$).

Dry matter and nitrogen bioconversion efficiency

Dry matter bioconversion efficiency (Table 4) responded strongly to addition timing. Adding excreta at 72 h decreased BE_{DM} by 18% (EE: 3.78%, LE: 3.10%; $p = 0.046$). In contrast, 72 h addition of glucose and starch increased BE_{DM} by 27% (EG: 4.57%, LG: 5.78%, $p < 0.001$) and 20% (ES: 4.06%, LS: 4.89%, $p = 0.036$), respectively. Cellulose addition at 72 h resulted in an 8% decrease in BE_{DM}

that was not significant (EC: 3.27%, LC: 3.01%, p = 0.944). Nitrogen bioconversion efficiency (Table 4) showed the same directional pattern but with larger relative changes. It decreased by 9% for 72 h excreta addition (EE: 6.70%, LE: 6.13%, p = 0.930) and increased by 36% for 72 h glucose addition (EG: 9.87%, LG: 13.43%, p < 0.001) and 37% for 72 h starch addition (ES: 8.10%, LS: 11.06%, p < 0.001). Cellulose addition at 72 h produced a 2% decrease in BE_N (EC: 6.91%, LC: 6.80%, p = 1.000).

Substrate mass loss and nitrogen emission

Pure excreta showed the highest and lowest wet mass loss of all substrates (EE: 45.9%, LE: 31.7 %, p < 0.001) (Table 4). In contrast, wet mass losses of glucose (EG: 36.7%, LG: 35.7%, p = 1.000), starch (ES: 39.9%, LS: 35.2%, p = 0.181) and cellulose (EC: 35.8%, LC: 33.7%, p = 0.914) were lower, with minimal differences between addition times (< 6% absolute change). Dry mass loss showed a different pattern: glucose (EG: 31.7%, LG: 22.4%, p = 0.050) and cellulose (EC: 28.5%, LC: 22.1%, p = 0.158) both lost ~30% less dry mass when added at 72 h. In contrast, dry mass losses remained nearly unchanged for excreta (EE: 20.8%, LE: 20.5%, p = 1.000) and starch (ES: 33.1%, LS: 32.3%, p = 1.000). Overall, 0 h glucose and both 0 h and 72 h starch additions caused more dry mass loss than 0 h and 72 h excreta (all contrast p < 0.05). When dry mass loss was expressed per unit larval dry matter, excreta (EE: 5.49 g/g, LE: 3.23 g/g, p = 0.223) and glucose (EG: 4.87 g/g, LG: 2.14 g/g, p = 0.284) showed the largest reductions between addition times. Nitrogen emission remained stable within each addition type. Excreta showed nearly identical values at 0 and 72 h (EE: 47.4%, LE: 48.2%, p = 0.999), as did glucose (EG: 47.4%, LG: 45.0%, p = 0.947) and starch (ES: 47.4%, LS: 46.1%, p = 0.999). Cellulose also showed no meaningful change over time (EC: 59.3%, LC: 58.9%, p = 1.000) but consistently produced the highest N emissions of all additions.

Table 4. Substrate wet and dry mass loss, dry mass loss per larval dry matter, nitrogen bioconversion efficiency (BE) and nitrogen emission of chicken excreta used as substrate for housefly larvae, with the addition of excreta, glucose, starch or cellulose at the start (early) or after 72 h (late).

Parameter	Substrate							
	Excreta		Glucose		Starch		Cellulose	
	Early	Late	Early	Late	Early	Late	Early	Late
Wet mass loss (%)	45.9 ^{a} ± 3.3	31.7 ^{b} ± 3.1	36.7 ^{c} ± 3.2	35.7 ^{c} ± 3.2	39.9 ^{d} ± 3.3	35.2 ^{cd} ± 3.2	35.8 ^{c} ± 3.2	33.7 ^{c} ± 3.1
Dry mass loss (%)	20.8 ^{a} ± 4.2	20.5 ^{a} ± 4.1	31.7 ^{b} ± 4.8	22.4 ^{a} ± 4.3	33.1 ^{b} ± 4.8	32.3 ^{b} ± 4.8	28.5 ^{b} ± 4.7	22.1 ^{a} ± 4.3
Dry mass loss/larval dry matter (g/g)	5.49 ^{a} ± 1.64	3.23 ^{b} ± 1.65	4.87 ^{a} ± 1.65	2.14 ^{b} ± 1.64	5.63 ^{a} ± 1.65	4.72 ^{a} ± 1.65	5.86 ^{a} ± 1.65	4.35 ^{a} ± 1.65
Dry matter BE (%)	3.78 ^{a} ± 0.34	3.10 ^{b} ± 0.31	4.57 ^{c} ± 0.37	5.78 ^{d} ± 0.42	4.06 ^{ac} ± 0.35	4.89 ^{cc} ± 0.39	3.27 ^{b} ± 0.31	3.01 ^{b} ± 0.30
Nitrogen BE (%)	6.70 ^{a} ± 0.67	6.13 ^{a} ± 0.64	9.87 ^{b} ± 0.79	13.43 ^{c} ± 0.92	8.10 ^{a} ± 0.73	11.06 ^{b} ± 0.84	6.91 ^{a} ± 0.68	6.80 ^{a} ± 0.67
Nitrogen emission (%)	47.4 ^{a} ± 2.7	48.2 ^{a} ± 2.7	47.3 ^{a} ± 2.7	45.0 ^{a} ± 2.7	47.4 ^{a} ± 2.7	46.1 ^{a} ± 2.7	59.3 ^{b} ± 2.6	58.9 ^{b} ± 2.6

Data presented as mean ± margin of error and based on n = 6. Means with different superscript letters within row differ significantly (Tukey-adjusted, $p \leq 0.05$).

Residue uric acid, ammonia, pH and C/N

In all substrates, uric acid content decreased by more than 90% (Table 5). Excreta (EE: 94.8%, LE: 94.0%, p = 0.960) and glucose (EG: 95.6%, LG: 95.6%, p = 1.000) showed no significant difference. Cellulose added at 72 h showed a small decrease in uric acid loss (EC: 97.3%, LC: 95.7%, p = 0.041), whereas loss increased for 72 h starch addition (ES: 95.5%, LS: 98.0%, $p < 0.001$). Residue NH_3 content increased up to 50% in residues compared to fresh substrate, with 72 h additions generally resulting in smaller increases (Table 5). Excreta addition at 72 h showed NH_3 content similar to 0 h (EE: 3.05 g/kg, LE: 2.97 g/kg, p = 1.000), lower than that of glucose additions (EG: 3.43 g/kg, LG: 3.36 g/kg, p = 1.000), which showed the largest increase of all additions. Starch showed the largest difference between addition times and the smallest increase for 72 h (ES: 3.11 g/kg, LS: 2.77 g/kg, p = 0.170) while cellulose also decreased slightly (EC: 3.00 g/kg, LC: 2.93 g/kg, p = 0.400). Residue pH increased for all additions compared to the fresh substrate (Table 5). Excreta addition showed the highest overall residue pH at 72 h and a clear increase between 0 and 72 h (EE: 8.61, LE: 8.91, p = 0.007). Cellulose had the overall lowest 0 h pH and the largest increase between 0 and 72 h (EC: 8.26, LC: 8.88, $p < 0.001$). Glucose addition yielded the smallest change (EG: 8.85, LG: 8.74, p = 0.806). Starch exhibited the lowest 72 h pH, with 0 h comparable to 0 h cellulose (ES: 8.30, LS: 8.55, p = 0.021). Residue C/N increased for all additions compared to the starting substrate (Table 5). Cellulose showed the highest C/N values (EC: 18.4, LC: 19.6, p = 0.361), much higher than excreta (EE: 13.5, LE: 13.3, p = 1.000) and starch (ES: 13.0, LS: 13.6, p = 0.988). Early glucose addition produced values similar to excreta and starch, but 72 h addition led to a substantially higher C/N (EG: 13.7, LG: 15.7, p = 0.054).

Table 5. Uric acid loss, ammonia content, pH and C/N of housefly larvae substrate residues, with the addition of excreta or glucose, starch or cellulose at the start (0 h, early) or after 72 h (late) to the chicken excreta substrate.

Parameter	Substrate							
	Excreta		Glucose		Starch		Cellulose	
	Early	Late	Early	Late	Early	Late	Early	Late
Uric acid loss (%)	94.8 [a] ± 0.8	94.0 [a] ± 1.0	95.6 [a] ± 0.8	95.6 [a] ± 0.8	95.5 [a] ± 0.8	98.0 [b] ± 0.5	97.3 [b] ± 0.7	95.7 [a] ± 0.8
Ammonia content (g/kg DM)	3.05 [a] ± 0.19	2.97 [a] ± 0.18	3.43 [b] ± 0.20	3.36 [b] ± 0.20	3.11 [a] ± 0.19	2.77 [c] ± 0.18	3.00 [a] ± 0.19	2.93 [a] ± 0.19
pH	8.62 [a] ± 0.11	8.91 [b] ± 0.11	8.85 [c] ± 0.10	8.74 [c] ± 0.10	8.30 [d] ± 0.10	8.55 [a] ± 0.11	8.26 [d] ± 0.10	8.88 [bc] ± 0.10
C/N	13.5 [a] ± 0.9	13.3 [a] ± 1.0	13.7 [a] ± 1.0	15.7 [b] ± 1.0	13.0 [a] ± 1.0	13.6 [a] ± 1.0	18.4 [c] ± 0.9	19.6 [c] ± 1.0

Data presented as mean ± margin of error and based on n = 6. Means with different superscript letters within row differ significantly (Tukey-adjusted, $p \leq 0.05$).

Factor Analysis results

The first dimension contained 33.2% of the total variance and was primarily defined by mass-related and bioconversion variables (Table 6). The BE_{DM}, BE_N, total larval weight showed strong positive correlations (r = >0.8). Individual larval weight, larval survival, larval C/N ratio and larval dry matter were moderate positive contributors (r = 0.3 to 0.8). Correlations of residual NH_3, substrate dry mass loss, substrate uric acid loss, substrate water loss and residue pH were negligible., In contrast, nitrogen emission, substrate dry mass loss per larva dry matter were moderately negatively correlated (r = -0.7 to -0.6). Addition was strongly associated (r = 0.78) with the first dimension, whereas addition time was not (r = 0.1). The second dimension contained 18.2% of variation and was predominantly defined by residue characteristics, with lower correlations than seen for dimension 1. Residual C/N, residual pH, larval survival, total larval weight and N emission were its dominant positive correlations (r = 0.4-0.8). The BE_N (r = 0.3) also contributed, while individual larval and uric acid loss were negligible as were residual NH_3, BE_{DM} and dry loss per larva dry matter. The strongest negative correlation with dimension 2 was shown by larval dry matter (r = -0.8) and substrate water loss (r = -0.5). Both addition (r = 0.6) and addition time (r = 0.3) showed significant positive correlations with this dimension.

Table 6. Correlation coefficients (r) and p-values for quantitative and qualitative variables with Dimension 1 (33.2%) and Dimension 2 (18.2%) of the factor analysis. Variables are sorted within each dimension by absolute correlation strength.

Dimension 1		Dimension 2	
Variable	**r/p**	**Variable**	**r/p**
BE_{DM}[1]	0.974 / <0.001	Residue C/N	0.773 / <0.001
BE_N[2]	0.933 / <0.001	Survival	0.464 / <0.001
Larval mass total	0.879 / <0.001	Addition[3]	0.621 / <0.001
Addition[3]	0.782 / <0.001	N emission	0.455 / 0.012
Individual larval mass	0.754 / <0.001	Larval mass total	0.437 / 0.018
Survival	0.697 / <0.001	Residue pH	0.415 / 0.003
Larval C/N	0.644 / 0.007	BE_N[2]	0.275 / 0.058
Larval DM	0.373 / 0.009	Addition time[3]	0.264 / 0.018
Residue NH_3	0.330 / 0.219	Individual larval mass	0.172 / 0.242
Addition time[3]	0.119 / 0.460	Substrate uric acid loss	0.132 / 0.368
Substrate dry matter loss	0.112 / 0.449	Residue NH_3	0.112 / 0.448
Substrate uric acid loss	0.107 / 0.469	BE_{DM}[1]	0.062 / 0.673
Substrate wet mass loss	0.095 / 0.521	Sub dry loss/larval dry	0.022 / 0.988
Residue pH	0.011 / 0.938	Substrate dry matter loss	-0.204 / 0.163
Residue C/N	-0.361 / 0.118	Larval C/N	-0.361 / 0.076
Nitrogen emission	-0.677 / <0.001	Substrate wet mass loss	-0.455 / 0.012
Sub dry loss/larval dry	-0.761 / <0.001	Larval DM	-0.804 / <0.001

[1] Bioconversion efficiency (dry matter). [2] Bioconversion efficiency (nitrogen).

[3] Qualitative variable.

Discussion

Larval growth and bioconversion

Optimizing larval growth and survival is key for bioconversion of excreta by housefly larvae. This study investigated whether larval instars differ in their use of available carbohydrates and how this affects growth and bioconversion. The effects of addition time within and between substrates do support the hypothesis that older larvae are better at utilizing easily digestible carbohydrates. Effects were specific to the carbohydrate used. Addition of starch at 72 h considerably increased both individual and total harvested larval mass compared to 0 h addition or addition of fresh excreta at both time points, whereas 0 and 72 h glucose addition both resulted in larger masses compared to pure 0 h excreta and 0 h starch addition. Addition of 72 h excreta negatively affected larval mass and survival. Cellulose, intended as an inert control for stirring that might affect larvae, did not have any time dependent effect on larval growth.

The time dependent effects of starch support the concept of a nutrient utilization shift of larvae. As stated in the introduction, a potential substrate of housefly amylases is bacterial glycogen, formed under conditions of stress by facultative anaerobic bacteria abundant in excreta such as *E. coli* and *Enterobacter* spp. (Wilson *et al*. 2010, Cifuente *et al*. 2019). Defecation leads to a die-off of the obligate anaerobic gut bacteria that provides ample soluble bacterial protein for larval nutrition, while glycogen producing bacteria could increase in abundance after this “proteinous” phase. The early

high availability of protein that supports early larval growth allows for the morphological changes in mouth parts and the development of enzymes (digestive lysozyme, amylases) needed for ingestion and digestion of bacteria by older larvae. But an alternative explanation is that glycogen producing bacteria are not needed for heightened amylase activity and amylases and consequent ability to digest starch are purely facultative. Housefly larvae, that do not need starch for completion of their larval stage, have a structural diversity in amylase genes allowing them to use different substrates and might only translate genes into enzymes when starch is available. Through their specialized mouth parts, late instars ingest and digest bacteria regardless of glycogen content. The small or absent effect of early starch addition on individual and total larval mass compared to early excreta might also be due to physical characteristics of starch, with early instar mouth parts unable to transport starch into the digestive tract. Starch is granulate in nature and has a relatively low solubility and high viscosity (Cornejo-Ramírez *et al*. 2018). This could explain the difference with glucose, that has a very high solubility and indeed showed strong positive effects on larval growth for early addition.

In the light of the high microbial activity of fresh excreta, the strong positive effects on larval growth of 0 h glucose addition were rather surprising. Unsterilized excreta harbours an array of microorganisms that potentially utilize glucose faster than growing housefly larvae. Simple sugars used as composting aid generate a fast and strong microbial metabolic response, reducing nitrogen availability by incorporation into bacterial mass (Meng *et al*. 2021). However, in this experiment larval growth and survival for 0 h glucose was better than in the 0 h excreta. The explanation for this might be sufficient emission of antimicrobial compounds by larvae, or suppression by of symbiotic microorganisms. Also, the rapid bagging and freezing of the excreta used might have decreased their exposure to airborne spores of relevant microorganisms or delaying their early growth.

The negative effect of 72 h excreta on both larval growth and survival might be due to the disruption of the optimal bacterial community. Excreta at 72 h led to a high pH of substrate residues but so did the better performing 0 h glucose, while its effect on the amount of NH_3 in residue was comparable to that of 0 h starch and 0 and 72 h cellulose. The 72 h addition of excreta might disrupt the growth of a specific microorganism needed by older larvae or increase immune stress by introducing the wrong bacteria species at the wrong time. A continuous feeding process, where larvae are fed with portions of fresh chicken manure, was found optimal for black soldier fly larvae bioconversion (Dzepe *et al*. 2021) but this seems a questionable feeding strategy for housefly larvae. From the larval dietary shift perspective, future studies are needed to determine larval growth in time and unravel the relation between larval density, microorganisms and dietary components. Inoculating fresh substrates with specific bacteria or providing young larvae with a different diet could be possible strategies to drastically improve larval yield and bioconversion. Specific neonate diets, higher in animal proteins, are used for neonates of *Hermetia illucens* in mass-rearing conditions (Woods *et al*. 2019). Combining a specific neonate diet with mass oviposition or artificial initial high larval density might solve the problem of low larval survival in mass-rearing conditions, as addressed by Čičková *et al*. (2013).

Compared to 0 h pure excreta, BE_{DM} and BE_N increased substantially by adding carbohydrates. BE_{DM} increased 30% for 72 h starch, 21% and 53% for 0 and 72 h glucose, while BE_N of 72 h starch increased by 65% and 47% and 100% for 0 and 72 h glucose, respectively. Bioconversion efficiencies of pure excreta (0 h) were similar to those found by Pisa *et al*. (2022) using excreta from the same source, notwithstanding this study's larger diet mass (500 g vs 100 g) and excreta homogenization. Despite the growing body of scientific publications on housefly bioconversion, to date no other studies were found reporting BE_N of manures by housefly larvae. Concerning BE_{DM} of pure chicken excreta, comparison to other studies is hindered by differences in experimental set-up (type and age of excreta, larval density, substrate mass), process end points (harvesting time, larval stage) and measured parameters (only reporting wet mass or relative larval growth). Disregarding these differences and using this study's substrate and larval DM, calculated dry matter bioconversion in literature ranges from 0.02% to 9.2% (Miller *et al*. 1974, Farkas *et al*. 1998, Cai *et al*. 2017, Hasan and Leong 2018, Miranda *et al*. 2020, 2023). The BE_{DM} found in this study is at the center of this range, with none of the carbohydrate additions increasing it above 5.78%.

Miranda *et al*. (2023) used a density of 1 larva / g growing in 1 kg of pure excreta found an average individual larval weight of 30 mg with a survival of 67%. They attributed the high individual weight to the large substrate mass which allowed for larval aggregates increasing performance. Combining their reported weights with the substrate and larval N content of this study results in BE_N of 8.8 to 13%, similar to the maximum of this study (13.5%). Increased survival of older larvae appears to be responsible for the found maximum, with 72 h glucose addition increasing survival of more but lighter (smaller) larvae at the used density of 8 larva/g substrate. Growing black soldier fly larvae on fresh pig feces, Parodi *et al*. (2021) reported a BE_N of 25%, roughly four times higher than that of pure chicken excreta used in this study. Apart from using a different fly and feces, they used larvae started on a different substrate and small amounts of feces (75 g) inoculating to 1.3 larva/ g wet substrate. A review on the use of BSF for bioconversion of manures was conducted by Grassauer *et al*. (2023) but the plethora of different rearing methods and general absence of reporting N content again hinders proper comparisons. Future research on both housefly and black soldier fly larvae bioconversion would benefit greatly from standardization, as proposed in Bosch *et al*. (2020).

Substrate nitrogen dynamics

Generation and emission of NH_3 are important and unwanted aspects of insect bioconversion of manures. Black soldier fly larvae in pig manure increased substrate ammonia content by 50 to 100% and emission of gaseous NH_3 by 60% compared to manure without larvae, with NH_3 making up 50% of the nitrogen lost during housefly larval growth on pig manure (Zhu *et al*. 2015, Chen *et al*. 2019, Parodi *et al*. 2021).

In this study, excreta and carbohydrate additions showed distinct effects on residue NH_3 content. Addition of 0 and 72 h glucose significantly increased the NH_3 content of the substrate residues compared to pure excreta. Potentially, glucose increased larval biomass by increasing survival and individual larval weight, with the resulting larger larval biomass excreting more nitrogenous waste products and bacteria converting this waste into NH_3. But this contrasts with relatively low substrate

uric acid loss associated with glucose additions, with the factor analysis showing uric acid not associated with larval mass but with drying of the substrate. This might indicate that glucose causes a difference in microbiota or changes larvae-bacteria interactions, possibly mediated by mechanical action and heat production. Addition of 0 and 72 h cellulose, 0 and 72 h excreta and 0 h starch resulted in lower and similar residue NH_3 content, yielding similar larval masses, in accordance with their metabolic unsuitability (cellulose) or wrong timing for either bacterial and/or larval growth. Starch addition at 72 h significantly reduced the increase in residue NH_3 content, while showing an increase in larval mass comparable to 0 h glucose. The potential explanation for its effect is that 72 starch fits to the time dependent nutritional demand of older larvae and thus decreases their production of nitrogenous waste that would lead to increased NH_3 concentrations. An alternative mechanism, microbial utilisation of N that incorporates NH_3 into bacterial biomass, is less likely as that should lead to a higher recovery of total N in starch substrate residues. This was not the case as N loss, so not recovered from residues or larvae, was highly similar for all timed addition of excreta, glucose and starch. Interestingly, the amount of N lost increased and was similar for 0 and 72 h cellulose additions compared to all other substrates. A possible explanation is that cellulose addition forced larvae to use protein as energy source without the change in microbiota brought on by 72 h addition of excreta. As all residue NH_3 measurements are taken at 120 h, there might be a time component responsible for the effect of cellulose, with most of the nitrogen lost via NH_3 volatilization in the early part of the growing period.

Including starch rich materials could decrease NH_3 emissions for housefly and black soldier fly bioconversion of manure. Adding carbohydrates to increase microbial utilization of nitrogen was suggested by Parodi *et al*. (2022) as a strategy to lower NH_3 emission from black soldier flies grown on pig manure, with fixation of nitrogen into both bacterial and larval biomass preventing its emission as NH_3. However, in this study no increase in total N content of 72 h starch substrate residues compared to the other additions was found. Factor analysis revealed that N emission was one of the key defining variables for dimension 2, where it clustered with residue characteristics such as pH and C/N. Larval survival, total larval weight and BE_N also contributed moderately to this axis, while individual larval weight and BE_{DM} were weakly associated. This indicates that residue nitrogen dynamics may be partially decoupled from individual larval mass and more closely linked to overall survival and microbial processes influenced by substrate composition.

Taken together, these findings suggest that ammonia dynamics and N loss are shaped by the interplay of larval metabolism, substrate microbial activity and nutrient availability. When appropriately timed, adding carbohydrates such as starch can reduce N losses without compromising yields. This aligns with Parodi *et al*. (2022) who advocate carbohydrate supplementation to promote microbial N assimilation and reduce NH_3 volatilization in insect-based manure treatment systems. However, the similarity of substrate N emission of 72 h starch addition in this study suggests that the mechanism may lie more in modulating larval excretion and N partitioning than in promoting microbial fixation.

Conclusions

This study demonstrates that adding carbohydrates (glucose, starch, cellulose) and the timing thereof affect growth and bioconversion efficiency of housefly larvae grown in chicken excreta. Glucose was found to be optimal for stimulating survival and growth in both early- and late-stage larvae. Interestingly, 72 h glucose addition showed stronger positive effects than 72 h starch addition and the 0 h glucose addition performed unexpectedly well, considering the high bacterial load of excreta. While less effective for early-stage larvae, 72h starch addition improved performance, likely aligning better with the nutritional needs of later-stage larvae. Early larval instars appear limited in ingesting or digesting starch, which improves as the larvae mature. The timing of carbohydrate addition was crucial, as both glucose and starch added at 72 hours enhanced larval survival, growth, total biomass and nitrogen upcycling from excreta, compared to 0 h addition. This emphasizes that the availability of digestible carbohydrates at the right time influences nitrogen retention and bioconversion from excreta into larval biomass. Excreta and cellulose additions, however, had minimal or negative impacts on growth, with excreta addition showing no clear effects and cellulose decreasing growth and bioconversion efficiency. Glucose addition increased NH_3 content in substrate residues, while the 72-hour starch addition reduced NH_3 accumulation.

References

Ayodele, T., Tijani, A., Liadi, M., Alarape, K., Clementson, C., Hammed, A. (2024). Biomass-based microbial protein production: A review of processing and properties. *Frontiers in Bioscience-Elite*, 16(4), 40.

Bates, D., Mächler, M., Bolker, B., Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, 67(1), 1-48.

Blahovec, J., Kostecká, Z., Kočišová, A. (2006). Peptidolytic enzymes in different larval stadium of housefly *Musca domestica. Veterinarni Medicina,* 51(4), 139-144.

Borchers, H. W. (2022). pracma: Practical Numerical Math Functions (Version 2.3.8) [Computer software]. R Foundation for Statistical Computing.

Bosch, G., Oonincx, D. G. A. B., Jordan, H. R., Zhang, J., van Loon, J. J. A., van Huis, A., Tomberlin, J. K. (2020). Standardisation of quantitative resource conversion studies with black soldier fly larvae. *Journal of Insects as Food and Feed,* 6(2), 95-110.

Brookes, F. J., Fraenkel, G. (1958). The nutrition of the larva of the housefly. *Physiological Zoology,* 31(3), 208-223.

Cai, Z., Yang, D., Wu, S., Wang, Y., Reaney, M. J. T., Zhao, Z., Zhu, L., Sun, G., Niu, Y., Zheng, D., Niu, H., Yang, W. (2017). Conversion of poultry manure to biodiesel, a practical method of producing fatty acid methyl esters via housefly (*Musca domestica* L.) larval lipid. *Fuel*, 210, 463-471.

Cribari-Neto, F., Zeileis, A. (2010). Beta Regression in R. *Journal of Statistical Software* 34(2), 1-24.

Chandra, G., Chater, K. F., Bornemann, S. (2011). Unexpected and widespread connections between bacterial glycogen and trehalose metabolism. *Microbiology,* 157(6), 1565-1572.

Chang, J. T., Wang, M. Y. (1958). Nutritional Requirements of the Common Housefly, *Musca domestica vicina* Macq. *Nature*, 181, 566-568.

Chen, J., Hou, D., Pang, W., Nowar, E. E., Tomberlin, J. K., Hu, R., Chen, H., Xie, J., Zhang, J., Yu, Z., Li, Q. (2019). Effect of moisture content on greenhouse gas and NH_3 emissions from pig manure converted by black soldier fly. *Science of the Total Environment* 697, 133840.

Čičková, H., Pastor, B., Kozánek, M., Martínez-Sánchez, A., Rojo, S., Takáč, P. (2012). Biodegradation of pig manure by the housefly, *Musca domestica*: A viable ecological strategy for pig manure management. *PLoS ONE*, 7(3), e32798.

Čičková, H., Kozánek, M., Takáč, P. (2013). Improvement of survival of the house fly (*Musca domestica* L.) larvae under mass-rearing conditions. *Bulletin of Entomological Research*, 103(1), 119-125.

Cifuente, J. O., Comino, N., Trastoy, B., D'Angelo, C., Guerin, M. E. (2019). Structural basis of glycogen metabolism in bacteria. *Biochemical Journal*, 476(14), 2059-2092.

De Jonge, N., Michaelsen, T. Y., Ejbye-Ernst, R., Jensen, A., Nielsen, M. E., Bahrndorff, S., Nielsen, J. L. (2020). Housefly (*Musca domestica* L.) associated microbiota across different life stages. *Scientific Reports*, 10(1), 7842.

Dzepe, D., Nana, P., Kuietche, H. M., Teguia, A. (2021). Feeding strategies for small-scale rearing black soldier fly larvae (*Hermetia illucens*) as organic waste recycler. *SN Applied Sciences*, 3, 252.

Elahi, U., Ma, Y., Wu, S., Wang, J., Zhang, Q., Qi, G. (2020). Growth performance, carcass characteristics, meat quality and serum profile of broiler chicks fed on housefly maggot meal as a replacement of soybean meal. *Journal of Animal Physiology and Animal Nutrition*, 104(4), 1075-1084.

Esteban-Torres, M., Ruiz, L., Rossini, V., Nally, K., van Sinderen, D. (2023). Intracellular glycogen accumulation by human gut commensals as a niche adaptation trait. *Gut Microbes*, 15(1), 2235067.

FAO (2023). FAO statistical database. Accessed May 2023.

Farkas, R., Hogsette, J. A., Börzsönyi, L. (1998). Development of *Hydrotaea aenescens* and *Musca domestica* (Diptera: Muscidae) in poultry and pig manures of different moisture content. *Environmental Entomology*, 27(3), 695-699.

Gadzama, I. U., Ndudim, R. K. (2019). Nutritional composition of housefly larvae meal: a sustainable protein source for animal production – a review. *Acta Scientific Agriculture*, 3, 74-77.

Glencross, B. D., Huyben, D., Schrama, J. W. (2020). The Application of Single-Cell Ingredients in Aquaculture Feeds - A Review. *Fishes*, 5(3), 22.

Grassauer, F., Ferdous, J., Pelletier, N. (2023). Manure valorization using black soldier fly larvae: A review of current systems, production characteristics, utilized feed substrates, and bioconversion and nitrogen conversion efficiencies. *Sustainability*, 15, 12177.

Hasan, H. A., Leong, K. P. (2018). Growth of *Musca domestica* (Diptera: Muscidae) and *Sarcophaga dux* (Diptera: Sarcophagidae) larvae in poultry and livestock manures: Implication for animal waste management. *Journal of Asia-Pacific Entomology*, 21(3), 880-884.

Hristov, A. N., Ropp, J. K., Grandeen, K. L., Abedi, S., Etter, R. P., Melgar, A., Foley, A. E. (2005). Effect of carbohydrate source on ammonia utilization in lactating dairy cows. *Journal of Animal Science*, 83(2), 408-421.

Hwangbo, J., Hong, E. C., Jang, A., Kang, H. K., Oh, J. S., Kim, B. W., Park, B. S. (2009). Utilization of house fly-maggots, a feed supplement in the production of broiler chickens. *Journal of Environmental Biology*, 30(4), 609-614.

ISO 6496 (1999). Animal feeding stuffs. Determination of moisture and other volatile matter content. International Organization for Standardization, Geneva, Switzerland.

ISO 16634-1 (2008). Food products - determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content - Part 1: Oilseeds and animal feeding stuffs. International Organization for Standardization, Geneva, Switzerland.

Kumari, A., Singh, K., Kayastha, A. M. (2012). α-Amylase: General properties, mechanism and biotechnological applications - A review. *Current Biotechnology*, 1(1), 98-107.

Lam, K., Babor, D., Duthie, B., Babor, E.-M., Moore, M., Gries, G. (2007). Proliferating bacterial symbionts on house fly eggs affect oviposition behavior of adult flies. *Animal Behaviour*, 74, 81-92.

Lam, K., Tsang, M., Labrie, A., Gries, R., Gries, G. (2010). Semiochemical-mediated oviposition avoidance by female house flies, *Musca domestica*, on animal feces colonized with harmful fungi. *Journal of Chemical Ecology*, 36(2), 141-147.

Lê, S., Josse, J., Husson, F. (2008). FactoMineR: An R package for multivariate analysis. *Journal of Statistical Software*, 25(1), 1-18.

Lemos, F. J. A., Terra, W. R. (1992). A high yield preparation of *Musca domestica* larval midgut microvilli and the subcellular distribution of amylase and trypsin. *Insect Biochemistry and Molecular Biology*, 22(5), 433-438.

Levinson, J. H. (1960). Food of housefly larvae. *Nature*, 188, 427.

Meng, L., Li, W., Zhang, S., Zhang, X., Zhao, Y., Chen, L. (2021). Improving sewage sludge compost process and quality by carbon sources addition. *Scientific Reports*, 11(1), 1319.

Miller, B. F., Teotia, J. S., Thatcher, T. O. (1974). Digestion of poultry manure by *Musca domestica*. *British Poultry Science*, 15(2), 231-234.

Miranda, C. D., Cammack, J. A., Tomberlin, J. K. (2020). Life-history traits of house fly, *Musca domestica* L. (Diptera: Muscidae), reared on three manure types. *Journal of Insects as Food and Feed*, 6(1), 81-90.

Miranda, C. D., Cammack, J. A., Tomberlin, J. K. (2023). Large-scale production of house fly, *Musca domestica* (Diptera: Muscidae), larvae fed 3 manure types. *Journal of Economic Entomology*, 116(4), 1102-1109.

Nahm, K. H. (2005). Factors influencing nitrogen mineralization during poultry litter composting and calculations for available nitrogen. *World's Poultry Science Journal*, 61(2), 238-255.

Nayduch, D., Burrus, R. G. (2019). Flourishing in filth: House fly–microbe interactions across life history. *Annals of the Entomological Society of America*, 110(1), 6-18.

Niode, N. J., Mahono, C. K., Lolong, F. M., Matheos, M. P., Kepel, B. J., Tallei, T. E. (2022). A review of the antimicrobial potential of *Musca domestica* as a natural approach with promising prospects to countermeasure antibiotic resistance. *Veterinary Medicine International*, 2022, 9346791.

Parodi, A., Gerrits, W. J. J., Van Loon, J. J. A., De Boer, I. J. M., Aarnink, A. J. A., Van Zanten, H. H. E. (2021). Black soldier fly reared on pig manure: Bioconversion efficiencies, nutrients in the residual material, greenhouse gas and ammonia emissions. *Waste Management*, 126, 674-683.

Parodi, A., Yao, Q., Gerrits, W. J. J., Mishyna, M., Lakemond, C. M. M., Oonincx, D. G. A. B., Van Loon, J. J. A. (2022). Upgrading ammonia-nitrogen from manure into body proteins in black soldier fly larvae. *Resources, Conservation & Recycling*, 182, 106343.

Pastor, B., Anabel, A. M., Rojo, S., Cickova, H., Kozanek, M., Takac, P. (2011). Effect of the size of the pupae, adult diet, oviposition substrate and adult population density on egg production in *Musca domestica* (Diptera: Muscidae). *European Journal of Entomology*, 108(4), 587-596.

Pastor, B., Velasquez, Y., Gobbi, P., Rojo, S. (2015). Conversion of organic wastes into fly larval biomass: Bottlenecks and challenges. *Journal of Insects as Food and Feed*, 1(3), 179-193.

Pimentel, A. C., Barroso, I. G., Ferreira, J. M. J., Dias, R. O., Ferreira, C., Terra, W. R. (2018). Molecular machinery of starch digestion and glucose absorption along the midgut of Musca domestica. *Journal of Insect Physiology*, 109, 11-20.

Pisa, L., Oonincx, D. G. A. B., Bosch, G., Hendriks, W. H. (2022). Bioconversion of fresh chicken excreta by housefly larvae (*Musca domestica* L.) in relation to excreta sterilisation and carbohydrate addition. *Journal of Insects as Food and Feed*, 8(10), 1107-1116.

Pisa, L. W., Oonincx, D. G. A. B., Bosch, G., Hendriks, W. H. (2024). Dose-response effects of starch in sterilised and unsterilised chicken excreta on *Musca domestica* larval growth and bioconversion efficiency. *Journal of Insects as Food and Feed*, 11(5), 895-906.

R Core Team (2024). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rehman, H., Vahjen, W., Kohl-Parisini, A., Ijaz, A., Zentek, J. (2009). Influence of fermentable carbohydrates on the intestinal bacteria and enteropathogens in broilers. *World's Poultry Science Journal*, 65(1), 75-90.

Saleh, H. (2020). Review on using housefly maggots (*Musca domestica*) in fish diets. *Journal of Zoological Research*, 2(1), 1-5.

Sekar, K., Linker, S. M., Nguyen, J., Grünhagen, A., Stocker, R., Sauer, U. (2020). Bacterial Glycogen Provides Short-Term Benefits in Changing Environments. *Applied and Environmental Microbiology*, 86(9), e00049-20.

Skidmore, P. (1985). The biology of the Muscidae of the world. *Series Entomologica*, 29, 1-550.

Sinha, M. (1975). Amylase activity in the midgut of *Sarcophaga Ruficornis* and *Musca Domestica*. *Entomologia Experimentalis Et Applicata*, 18(3), 290-296.

Smith, L. W., Wheeler, W. E. (1979). Nutritional and Economic Value of Animal Excreta. *Journal of Animal Science*, 48(1), 144-156.

Strange, R. (1968). Bacterial "glycogen" and survival. *Nature*, 220, 606-607.

Szpila, K., Pape, T. (2008). Morphology of the first instar of the house fly *Musca domestica* (Diptera: Muscidae). *Journal of Medical Entomology*, 45(4), 594-599.

Terra, W.R., Espinoza-Fuentes, E.P., Ferreira, C. (1988). Midgut amylase, lysozyme, aminopeptidase and trehalase from larvae and adults of *Musca domestica*. *Archives of Insect Biochemistry and Physiology*, 9, 283-297.

Van Huis, A., Oonincx, D.G.A.B., Rojo, S., Tomberlin, J.K. (2020). Insects as feed: House fly or black soldier fly? *Journal of Insects as Food and Feed*, 6, 221-229.

Wang, Y., Yang, L., Zhang, Y., Tao, L., Wang, J. (2018). Development of *Musca domestica* at constant temperatures and the first case report of its application for estimating the minimum postmortem interval. *Forensic Science International*, 285, 172-180.

Weatherburn, M.W. (1967). Phenol hypochlorite reaction for determination of ammonia. *Analytical Chemistry*, 39, 971-974.

Weurding, R. E., Veldman, A., Veen, W. A. G., van der Aar, P. J., Verstegen, M. W. A. (2001). Starch digestion rate in the small intestine of broiler chickens differs among feedstuffs. *Journal of Nutrition*, 131(9), 2329-2335.

Wilson, W.A., Roach, P.J., Montero, M., Baroja-Fernández, E., José Muñoz, F., Eydallin, G., Viale, A.M., Pozueta-Romero, J. (2010). Regulation of glycogen metabolism in yeast and bacteria. *FEMS Microbiology Reviews*, 34(6), 952-985.

Woods, M.J., Hoffman, L.C., Pieterse, E. (2019). Artificial diets for neonatal black soldier fly (*Hermetia illucens*) larvae. *Journal of Insects as Food and Feed*, 5(2), 99-106.

Zhang, Z.J., Wang, H., Zhu, J., Suneethi, S., Zheng, J.G. (2012). Swine manure vermicomposting via housefly larvae (*Musca domestica*): the dynamics of biochemical and microbial features. *Bioresource Technology*, 118, 563-571.

Zhang, Q., Wang, S., Zhang, X., Zhang, K., Li, Y., Yin, Y., Zhang, R., Zhang, Z. (2022). Beneficial bacteria in the intestines of housefly larvae promote larval development and humoral phenoloxidase activity, while harmful bacteria do the opposite. *Frontiers in Immunology*, 13, 938972.

Zhao, Y., Wang, W., Zhu, F., Wang, X., Wang, X., Lei, C., Wang, H. (2017). The gut microbiota in larvae of the housefly *Musca domestica* and their horizontal transfer through feeding. *AMB Express*, 7, 147.

Zhu, F.-X., Yao, Y.-L., Wang, S.-J., Du, R.-G., Wang, W.-P., Chen, X.-Y., Hong, C.-L., Qi, B., Xue, Z.-Y., Yang, H.-Q. (2015). Housefly maggot-treated composting as a sustainable option for pig manure management. *Waste Management*, 35, 62-67.

Supplementary material

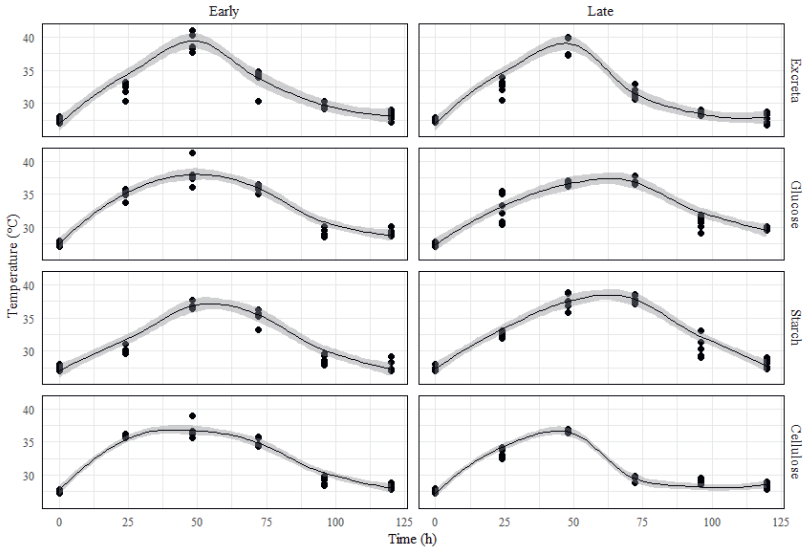


Figure S1. Temperature profile of chicken excreta used as substrate for housefly larvae, with the addition of excreta, glucose, starch or cellulose at the start (early) or after 72 h (late).

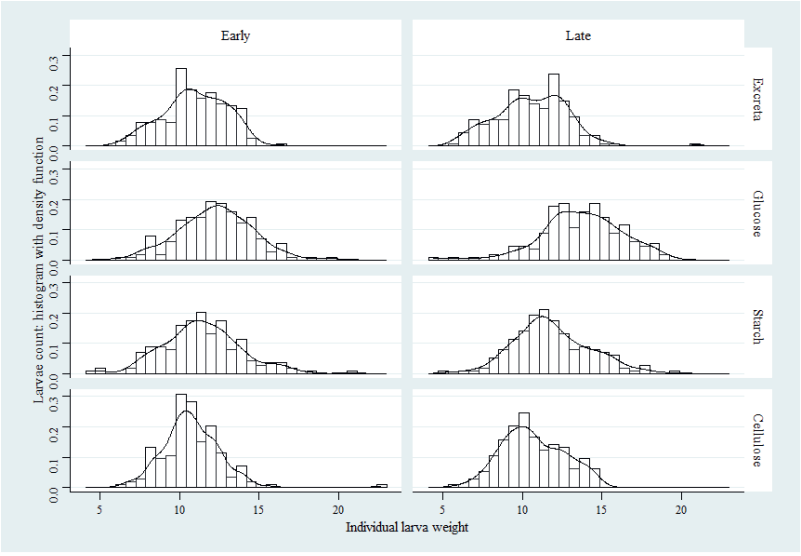


Figure S2. Histograms of individual larva wet mass, grown on of chicken excreta with the addition of excreta, glucose, starch or cellulose at the start (early) or after 72 h (late).

Chapter 5

Rearing of housefly larvae (*Musca domestica* L.) in different larval densities on chicken excreta supplemented with digestible carbohydrates

L.W. Pisa, D.G.A.B. Oonincx, W.H. Hendriks, G. Bosch

Submitted to Journal of Insects as Food and Feed

Abstract

Housefly larvae (*Musca domestica* L.) can convert livestock manure into protein-rich biomass, addressing waste management and feed sustainability challenges. This study examined how carbohydrate supplementation (glucose or starch) to chicken excreta and larval density affected time dependent larval growth, survival and bioconversion efficiency. A 3×4×2 factorial design tested three substrates (unsupplemented, glucose- and starch-supplemented chicken excreta), four egg densities (8-20 eggs/g substrate) and two harvest times (58 and 120 h). Glucose and starch increased early larval survival, larval biomass and bioconversion efficiency, with glucose showing more consistent effects. Higher densities improved survival, particularly in supplemented substrates, but accelerated substrate drying which reduced late-stage larval growth and bioconversion. Starch lowered residue ammonia and pH, suggesting better nitrogen retention. Factor analysis revealed that early-stage growth was more associated with substrate, whereas late stage growth was more associated with density. While carbohydrate supplementation and higher densities enhanced early-stage growth, substrate drying posed a challenge for late-stage larval biomass and bioconversion. Optimizing moisture and supplement timing could improve late-stage outcomes. These findings contribute to sustainable manure bioconversion strategies for housefly based feed production.

Introduction

Human demand for animal-derived foods has steadily increased and is expected to continue rising (FAO 2011, Baldi and Gottardo 2017). Greenspoon *et al*. (2023) estimated the total biomass of domesticated livestock at 630 million tonnes, vastly exceeding the 20 million tonnes of wild terrestrial mammals. To sustain this livestock, about 40% of arable land is used for feed crops such as soy in addition to 23% of captured fish being processed into feed for aquaculture, pigs, and poultry (Mottet *et al*. 2017, FAO 2020). Expanding or even maintaining livestock production is becoming increasingly difficult as arable land has declined by over 30% in the past 40 years and fish stocks face overexploitation (Britten *et al*. 2021, Prăvălie *et al*. 2021). Current livestock production has also drastically increased surplus manure in various countries, leading to environmental issues with greenhouse gas emissions, eutrophication and contamination of soil and water (Cai *et al*. 2021, Khoshnevisan *et al*. 2021).

Manure bioconversion by housefly (*Musca domestica* L.) larvae could offer a partial solution to both challenges, converting manure into nutrient-rich larval biomass while reducing its volume and improving biochemical characteristics of residues for use as fertilizer (Newton *et al*. 2005, Van Huis 2019, Heuel *et al*. 2021). The housefly is increasingly studied for bioconversion of animal manures, showing promising results (e.g. Čičková *et al*. 2012, Hussein *et al*. 2017, Miranda *et al*. 2020). Houseflies have a high reproduction rate and a short larval development time, with larvae combining a high crude protein content (50 to 60%) with a balanced amino acid profile and a relatively high lipid content (10-23%) (Gadzama and Ndudim 2019, Sánchez *et al*. 2021, Boatta *et al*. 2023). Fresh and dried larvae and larvae meals have been used successfully as diet ingredient for fish, poultry and crustaceans, with the potential to fully or partly replace soy and fish meal (Pieterse and Pretorius 2014, Fawole *et al*. 2023).

The effects of manure characteristics, including nutrient content and microbial abundance, on larval growth and survival remain poorly understood. Larvae are thought to ingest bacteria, with symbiotic microbial species thought to play nutritive and non-nutritive roles, e.g. reducing competition with yeasts (Levison 1960, Lam *et al*. 2009, Nayduch and Burrus 2017). Larval instars differ significantly in morphology of mouth parts (Szpila and Pape 2008), indicating adaptations to potential distinct dietary compositions and physical structures during larval development.

A key knowledge gap with implications for manure bioconversion by larvae is the role of carbohydrates. Though larvae can grow on a diet without sugars or starch, the presence of various gut amylases suggests they can utilize starch-like carbohydrates (Brookes and Frankel 1958, McCommas and Shornick 1990, Pimentel *et al*. 2018). Fresh manures contain little digestible carbohydrates (Svihus 2014, Rama and Tongol 2024) and in line with the presence of gut amylases, starch (up to 15% of substrate DM) addition to chicken excreta increases larval size, total biomass and overall dry matter (DM) and nitrogen (N) bioconversion (Pisa *et al*. 2022, 2024). The dietary presence of a digestible carbohydrate is also likely to affect larval N utilization efficiency and substrate ammonia (NH_3) emissions. When larvae utilize protein for energy production, their nitrogenous waste (uric acid) increases, which bacteria can convert to NH_3 that is easily lost through volatilization (Weihrauch

and O'Donnell 2021). Supplying digestible carbohydrates may reduce larval protein use for energy and, thereby, decrease uric acid excretion and NH_3 formation. Decreasing the dietary protein to energy ratio to lower N excretion has been a viable strategy in pigs, poultry and other animal species to increase production efficiency and ensure carcass quality characteristics (Cahn 1998, Wu *et al*. 2018). In addition, a substrate carbohydrate surplus may support bacterial assimilation of NH_3 into microbial biomass, further reducing emissions (Parodi *et al*. 2021, Huang *et al*. 2022). However, in contrast to these positive effects, all levels of starch addition (5 to 50% of substrate DM) reduced larval survival compared to pure excreta (Pisa *et al*. 2022, 2024). This may be explained by the fact that (excess) carbohydrates can facilitate detrimental microbial shifts or alter biochemical characteristics of substrates that negatively affect larval survival.

Larval growth and survival are strongly influenced by larval density with higher larval densities of housefly and black soldier fly (*Hermetia illucens*) larvae associated with lower body mass and lower survival through competition for resources (Opare *et al*. 2022, Grossule 2024). But, most muscoid flies including the housefly practice mass-oviposition, resulting in localized high initial larval densities that benefit early survival and growth. Higher larval densities allow larvae to form clusters that improve larval digestion and immune function by temperature increases in the larval mass, increasing activity of internal and secreted enzymes (Scanvion *et al*. 2018, Kortsmit *et al*. 2023). Effects of larval density on housefly life traits have been studied, but different study designs and substrates showed different optimal densities for growth and manure degradation (Calvert *et al*. 1970, Miller *et al*. 1974, Barnard *et al*. 1998, Moon *et al*. 2001, Hussein *et al*. 2017, Cai *et al*. 2017).

Here, the effect of larval density on larval survival and growth in chicken excreta supplemented with two highly bioavailable carbohydrate sources (starch or glucose) was investigated. As starch is a complex carbohydrate requiring enzymatic breakdown before absorption, its bioavailability is potentially dependent on larval density. In contrast, glucose is directly absorbable, providing immediate energy without the need for density dependent enzymatic breakdown, thus serving as a positive control. The central hypothesis was that starch addition reduces larval survival and growth, particularly at low larval densities, whereas higher densities mitigate this negative effect. Positive effects of higher larval density are expected to be greater for younger larvae, which may depend more on collective larval mass for efficient digestion. To test this, starch or glucose was added to chicken excreta at the start of the larval period, and larvae were harvested at two time points to assess early and full growth. Larval growth and substrate parameters were used to calculate larval density effects and the impact of carbohydrate supplementation on bioconversion, water and DM loss, NH_3 content and pH of the excreta substrates.

Materials and methods

Experimental design

The experiment combined three experimental substrates, four egg densities (ED) and two different harvest times (3×4×2 factorial design). Experimental substrates consisted of chicken excreta without or with a source of digestible carbohydrate (gelatinized corn starch or glucose). Applied ED were ~8,

12, 16 and 20 eggs/g wet substrate. At 58 or 120 h after inoculation, all larvae were harvested and substrate residues processed as specified below. Six replicates were made for all 24 experimental treatments (n = 6, N = 144) in six series. Each series always contained six samples of the same substrate-ED combination, consisting of three replicates per harvest time (three equal time pairs). Random assignment (without replacement) of samples to series was done using the R package "blockrand" (Snow 2020).

Housefly egg production

A housefly culture (Dutch GK strain, Groningen University, the Netherlands) was set up at Wageningen University & Research in August 2022 and experiments were conducted in December 2022. Adult flies were kept in mesh Bugdorm™ cages of 47.5×47.5×93 cm (MegaView Science Co., Taichung, Taiwan), furnished with cardboard sheets to increase the surface area. Cages contained ~3000 individuals per cage which were maintained at 27 °C, 60% RH and 16:8 light-darkness. Adult flies were provided ad libitum with a thin layer of milk-based baby formula (0 to 6 months standard, Albert Heijn home brand, Zaandam, the Netherlands) and sucrose cubes (Albert Heijn home brand, Zaandam, the Netherlands) on disposable plastic plates. Tap water was provided in polypropylene pots with cotton wicks inserted through the lids. Fresh food and water were provided every 48 h. Larvae were reared in the same room in 1500 ml black food grade polypropylene trays without lids (RE180 series, Disposable Discounter, Groningen, the Netherlands). Larval substrate was composed of one part dry extruded dog kibble (Albert Heijn adult lamb and rice, Partner in Pet Food, Doetinchem, the Netherlands), soaked in two parts water and covered with 1.5 parts (by weight) of dry wheat bran (Meneba, Rotterdam, the Netherlands). Adult flies were allowed to oviposit on a single tray with fresh substrate and young larvae were partitioned into new substrate trays at 400-500 larvae to ~300 g fresh substrate. During pupation, trays were placed in clean cages for eclosion. Eggs were collected from adult flies 13-15 days after eclosion with mixing of eggs of multiple cages and partitioned by weight into the substrates following the same procedure as described by Pisa *et al*. (2022). The average weight of a single housefly egg was determined to be 0.06 mg by weighing and manual counting (~50 mg of eggs three times). This average was used to weigh portions of eggs corresponding to 8, 12, 16 and 20 eggs/g wet substrate, with 159, 238, 317 and 396 mg eggs matching approximately 2640 (8 eggs/g), 3960 (12 eggs/g), 5280 (16 eggs/g) and 6600 eggs (20 eggs/g) for 330 g substrate. Based on substrate DM, these densities translated to 26, 40, 53 and 66 eggs/g substrate DM.

Experimental substrates

Fresh (<24 h old) excreta of layer hens fed a commercial wheat-soybean based feed were obtained from a commercial farm (Rondeel, Barneveld, the Netherlands) and homogenized using a FGC 10-2 bowl cutter (Feuma Gastromaschinen GmbH, Gößnitz, Germany). Visible feathers and egg shells were manually removed during homogenization, whereafter 1 kg portions were frozen in polyethylene Ziploc bags with concurrently small samples (6-8 g material) taken for rapid moisture content determination and chemical analyses. Moisture content of excreta was determined in sextuple while moisture content of gelatinized corn starch (Research Diet Services, Wijk bij Duurstede, the

Netherlands) and glucose (Sidroga Pharma, Bad Ems, Germany) were determined in triplicate (5 g material) with a MB 120 moisture analyzer (OHAUS Europe GmbH, Nänikon, Switzerland). Mean ± SD DM content of glucose and starch were 990.4 ± 0.4, 899.4 ± 1.9 g/kg, respectively. Based on ingredient DM content, substrate replicates were made to contain~100 g dry mass at ~30% DM, resulting in ~330 g substrate per tray. For the carbohydrate-containing substrates, starch (~10.8 g) or glucose (~10.0 g) were added as 10% of substrate total DM (see below). The pH of the substrates was measured in triplicate with a PHB-4 meter (INESA, Shanghai, China) just before egg inoculation. Chemical composition of excreta and ingredients as well as chemical composition of the three substrates are provided in Table 1 and 2.

Experimental procedures

Before substrate formulation, the excreta were thawed overnight to 5 °C before being weighed and transferred into 1.5 L (180×133×93 mm) transparent food grade polypropylene trays (RE180 series, Disposable Discounter, Groningen, the Netherlands). Glucose and starch were gently stirred into the excreta by hand using a fork into a uniform layer of approximately 5 cm deep in the trays, with the unsupplemented excreta substrate (UE) also similarly stirred. Trays with substrates were allowed to equilibrate to room temperature before being inoculated with eggs. Inoculated substrates were kept in the dark on shelves covered by a plastic sheet under the same conditions as used for the housefly culture.

Harvesting and processing of larvae and residues

During the 120 h larval growth period, surface temperature of the substrates was measured daily between 10:00 and 13:00 in the middle of the tray using an infrared thermometer (Ebro TFI 260, Xylem Analytics, Ingolstadt, Germany). At harvest time, trays were weighed and placed on ice water prior to larvae and substrate residue processing. After gentle stirring of the residues, their pH was measured and small portions (5 g) were freed from larvae for NH_3 analysis. Larvae were harvested by floatation in a 14% NaCl solution after which larvae and residues were processed and sampled as described by Pisa *et al*. (2022). Larvae and residues were subsequently frozen at -20 °C and freeze-dried before being ground over a 1 mm screen at 12000 rpm in a centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) and storage in polyethylene pots at room temperature pending chemical analyses.

Chemical analyses

Ammonia was determined in fresh substrate residues without larvae using a colorimetric method after deproteinisation with trichloroacetic acid, based on Weatherburn (1967). The pH was determined in fresh material using a PHB-4 meter (INESA, Shanghai, China). Dry matter (ISO 6496, 1999) and total N and carbon (C) by the Dumas method (ISO 16634-1, 2008) were analyzed in freeze dried material. Analyses of NH_3, N and C were performed in duplicate and DM performed in single measurements.

Calculations and statistical analyses

Total heat production was estimated as the area under the temperature curves using the trapezoidal integration function from the R pracma package (Borchers 2024). Means and confidence intervals of heat production were calculated with a linear model using substrate and ED as factors. Dry matter and N bioconversion efficiencies (BE_{DM}, BE_N, respectively) were calculated per tray as the absolute amounts (g) of DM and N in the total larval biomass divided by the absolute amount present in the larval substrates. Substrate water loss was calculated per tray as the absolute amount (g) of water in the substrate minus the absolute amount of water in the substrate residue (water lost), divided by the absolute amount of water in the substrate. Dry matter loss was calculated per tray as the absolute amount (g) of substrate DM minus the absolute amount of substrate residue DM, divided by the absolute amount of substrate DM.

Using a sample size of 30 larvae per tray, gamma-log link generalized linear models (GLM) improved error normality by accounting for zero-bound data and right skew (rare occurrence of heavy larvae). Mean individual larva weight was estimated using a full factorial model with substrate, ED and harvest time as fixed factors and "series" (day made) and "tray" as random intercepts. Despite their negligible effects (ANOVA, $p \sim 1.0$), the random factors were retained as they were part of the design and data size (4320 observations across 144 trays in 6 series) allowed for the used degrees of freedom. To incorporate non-normality of individual larval weight in survival estimates, a mean, low and high larval count were calculated per tray by dividing total wet larvae weight by the mean and 95% confidence limits of the gamma-distributed individual larva weight (Pisa *et al*. 2022). Assuming full egg survival and eclosion, larval survival was modelled using a binomial-logit link GLM with substrate, ED and harvest time as fixed factors in a full factorial design, with "series" as a random intercept and the ratios of low, mean and high larval count to number of eggs as dependent variable. Means and confidence intervals of continuous fractions (bioconversions, mass losses, NH_3 content) were estimated with beta-logit link GLM with substrate, ED and harvest time as factors in a full factorial design and "series" as a separate factor.

Means of total larvae wet weight and pH were estimated with linear mixed models with substrate, ED and harvest time as fixed factors in a full factorial design and "series" as random intercept. Model residuals were visually inspected for normality and patterns. Statistical inference was based on confidence intervals, with means outside the 95% confidence limits considered significantly different ($p < 0.05$). Models were ran using the R packages "betareg" and "lme4" in R version 4.3.12 (Cribari-Neto and Zeileis 2010, Bates *et al*. 2015, R Core Team 2023). To find associations between qualitative (substrate, ED) and quantitative variables (larval masses, survival, substrate mass losses, bioconversions, residue NH_3 and pH) not apparent in regression models, a factor analysis for mixed data was conducted separately for each harvest time using the R package FactoMineR, using the raw (unscaled) data (Lê *et al*. 2008).

Table 1. Mean ± SD analyzed composition (g/kg dry matter) of chicken excreta.

Item[1]	Excreta
Dry matter (% as is)	497 ± 3.7
Total carbon	366 ± 8.8
Total nitrogen	53.6 ± 2.6

[1] N = 6 for dry matter and N = 3 for other items.

Table 2. Ingredient (g) and calculated chemical composition (g/kg dry matter) of the three experimental substrates.

Item	Substrate		
	Excreta	Excreta + glucose	Excreta+starch
Ingredients per tray			
Chicken excreta	200	180	180
Glucose	0	10.0	0
Starch	0	0	10.8
Water	130	140	140
Chemical constituents			
Dry matter (g/kg as is)	303	303	303
Starch	0	0	100
Glucose	0	100	0
Ammonia[1]	2.3	2.1	2.1
pH	7.85	7.81	7.84
Carbon/nitrogen[1]	6.4	7.0	7.0

[1] Based on total material used and estimated dry matter content.

Results

The starting pH of the three substrates was similar (Table 2) and the addition of glucose or starch to the chicken excreta did not affect substrate consistency from tactile perception and visual observation. Distinct temperature profiles during larval growth were observed (Figure S1). The UE reached its highest temperature at 48 h (mean 40.8, max 41.9 °C) before rapidly decreasing, with greater ED maintaining slightly higher temperatures. Glucose-supplemented excreta (GSE) temperature also peaked at 48 h (mean 38.4, max 39.5 °C) but decreased slower after their peak values. In contrast, starch-supplemented excreta (SSE) had a lower peak temperature (mean 34.9, max 36.4 °C) at 72 h and a faster decrease. Total heat produced during 120 h larval growth was significantly greater for higher ED in UE and GSE but not SSE (Figure S2). Total heat produced of the latter was significantly lower compared to UE and GSE (all SSE means lower than lower 95% CI limits of UE and GSE).

Larval growth and survival

Larvae showed the greatest increase in mean individual weight during the first 58 h, accounting for 70-90% of total growth across substrates (Figure 1A). At both harvest times, mean individual weight decreased with increasing ED, most sharply between ED8 and ED12, with larvae on GSE and SSE

being consistently heavier than those on UE. On the UE, mean individual weight declined from 9.3 mg at ED8 to 5.8 mg at ED20 at 58 h (-38%) and from 10.6 mg to 5.3 mg at 120 h (-50%), leveling off beyond ED16 at both times. In the GSE, weights decreased from 10.9 mg to 6.2 mg at 58 h (-43%) and from 13.3 mg to 6.5 mg at 120 h (-51%), while in the SSE they declined from 11.3 mg to 5.9 mg (-48%) and from 14.0 mg to 7.2 mg (-49%) at 58 h and 120 h, respectively. Overall, larvae in supplemented substrates were on average 20-30% heavier than those in the UE, with differences increasing over time.

Increasing egg density significantly affected total larval wet weight in both GSE and SSE at 58 and 120 h (Figure 1B). On the UE, total larval wet weight changed little with density at 58 h, increasing only slightly from 24.1 g at ED8 to 26.1 g at ED20 (+8%), and more noticeably at 120 h, from 21.8 g to 27.0 g (+24%). In contrast, the GSE showed a strong linear increase with density, rising from 26.0 g to 38.3 g at 58 h (+47%) and from 19.9 g to 33.7 g at 120 h (+69%). The SSE followed a similar pattern, increasing from 27.3 g to 40.7 g at 58 h (+49%) and from 20.3 g to 31.1 g at 120 h (+53%), but with less linearity, showing plateaus between ED12-16 at 58 h and beyond ED16 at 120 h.

Larval survival on the GSE and SSE increased with ED at both harvest times, whereas survival on the UE remained largely constant or slightly declined (Figure 1C). On the UE, survival stayed stable across densities at 58 h (≈ 80%) and dropped modestly to ≈ 74% at 120 h, with little variation up to ED16 and a slight decrease from 76% to 71% at the highest density. In contrast, survival on the GSE rose from 72% to 89% at 58 h (+24%) and from 60% to 85% at 120 h (+42%), displaying a near-linear increase that leveled off between ED12 and 16. Survival on the SSE also improved with density, increasing from 69% to 84% at 58 h (+22%) and from 63% to 74% at 120 h (+17%), though the pattern was less consistent than on GSE.

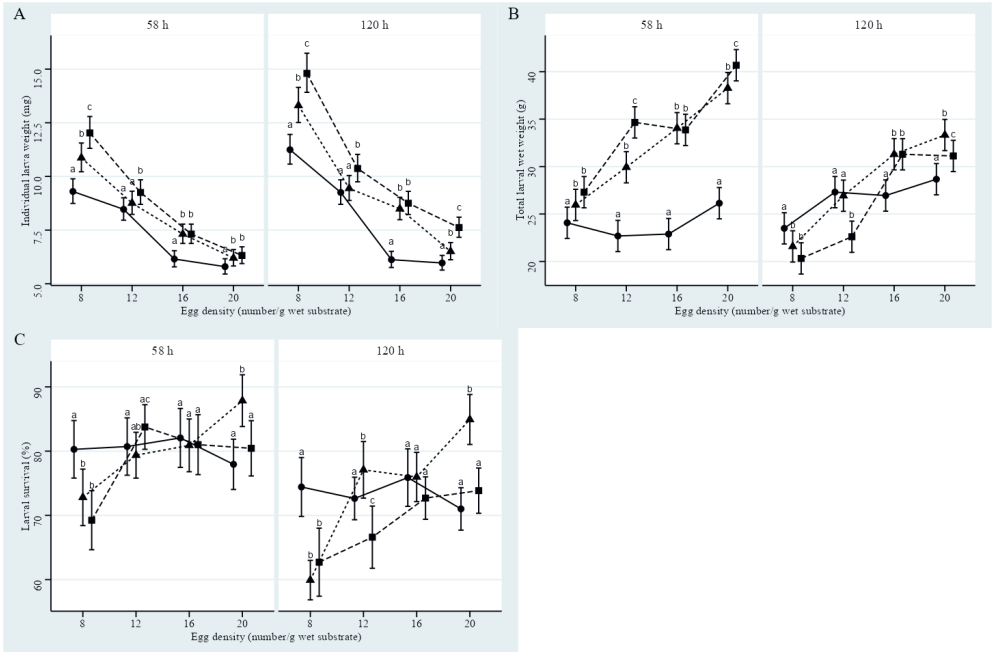


Figure 1. Housefly individual larva wet weight (A), total larvae wet weight (B) and larval survival (C) grown on excreta substrates without (none, ●) or with glucose (▲) or starch (■) addition, inoculated at different egg densities and harvested at 58 or 120 h. Symbols with whiskers show means and 95% confidence intervals. Means denoted by a different letter within egg density indicate significant differences between substrates. Data points were slightly offset along the x-axis to prevent overlap.

Dry matter and nitrogen bioconversion efficiency

The BE_{DM} increased with ED in both supplemented substrates at 58 and 120 h, though overall values were lower at 120 h (Figure 2A). For the UE, BE_{DM} declined slightly with increasing ED at 58 h, from about 5.7 to 5.0%, and then rose again at ED20, while at 120 h it increased modestly across densities by roughly 30%. In contrast, the GSE showed a small but consistent rise from 5.1 to 5.7% at 58 h (+12%) and a stronger increase from 3.8 to 5.6% (+47%) at 120 h with gains becoming smaller at higher densities. The BE_{DM} for SSE followed a nearly identical pattern at 58 h, increasing from 5.0 to 5.9% (+18%), but showed only a weak linear rise at 120 h, from 4.5 to 4.9% (+9%). Patterns in BE_N closely mirrored those of BE_{DM} (Figure 2B). At 58 h, BE_N for the GSE increased slightly from 9.7 to 11.0% (+13%) and for the SSE from 9.5 to 11.6% (+22%) while for UE it decreased from 6.7 to 5.2% (-22%). At 120 h, BE_N of UE rose from 5.0 to 6.6% (+32%) but remained well below the SSE (8.3-9.1%) and GSE (7.2-10.6%) values. Overall, carbohydrate supplementation maintained higher BE_{DM} and BE_N across densities and harvest times, with the strongest improvements in GSE, particularly at 120 h.

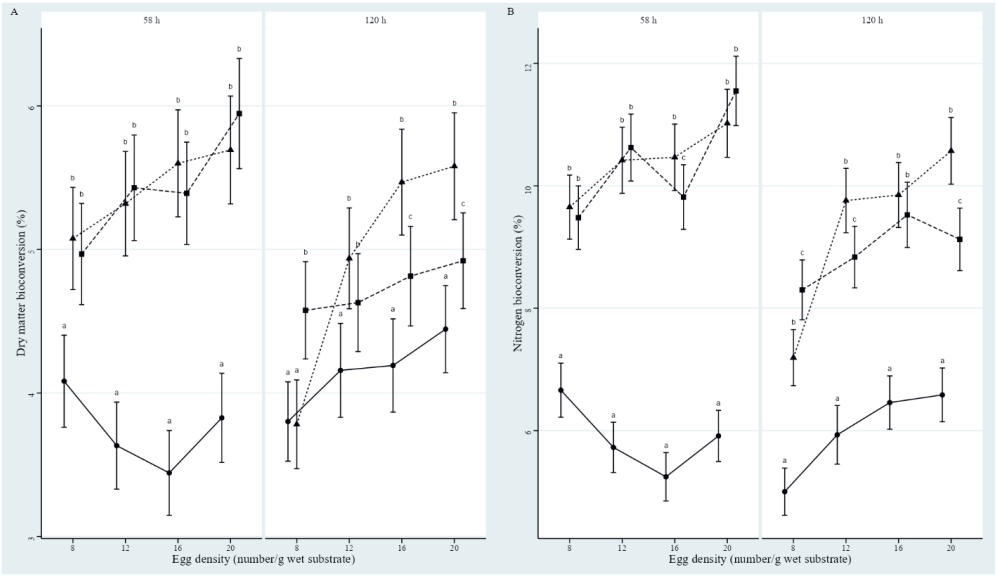


Figure 2. Dry matter (A) and nitrogen (B) bioconversion efficiency of housefly larvae grown on excreta substrates without addition (none, ●) or with glucose (▲) or starch (■), inoculated at different egg densities and harvested at 58 or 120 h. Symbols with whiskers show means and 95% confidence intervals. Means denoted by a different letter within egg density indicate significant differences between substrates. Data points were slightly offset along the x-axis to prevent overlap.

Substrate water and dry matter loss, residue NH_3 and pH

Water and dry matter losses increased with larval density and harvest time (Figure 3A-B). At 58 h, water loss rose weakly with ED up to ED16 and was similar for the GSE and SSE (25-33%), both significantly higher than the UE (18-22%). At 120 h, water loss increased further, maintaining the same positive relation with ED, with GSE and SSE (48-56%) exceeding the UE (33-39%). Dry matter loss showed comparable trends but stronger responses to ED. At 58 h, all substrates displayed weak positive relations with ED, with GSE and SSE (4-7%) exceeding the UE (2-5%). At 120 h, these relations became more pronounced, especially above ED12, with the SSE (8-15%) showing higher losses than GSE (7-13%) and both exceeding the nearly constant UE (10-11%). Residue NH_3 contents were higher at 58 h than at 120 h for all substrates (Figure 3C). At 58 h, residue NH_3 contents were highest in the UE (3.2-4.0%), showing a positive trend with ED (+25%) while in the GSE (4.0-3.7%) values declined slightly after ED8 (-8%). The SSE (3.5-3.8%) showed little variation across densities. At 120 h, NH_3 contents decreased overall, with the UE (2.4-2.9%) and the GSE (2.4-2.8%) showing positive relations with ED (+20%), while SSE (2.0-2.2%) remained lowest and unaffected by ED.

At 58 h, residue pH was highest in the UE (8.58-8.74) and GSE (8.05-8.14), both showing weak positive trends with ED (+2%), while the SSE (7.97-7.90) decreased slightly at higher ED (-1%). At 120 h, pH was again highest in the UE (8.63-8.79) and GSE (8.45-8.65), both increasing slightly with ED (+2%), whereas the SSE (8.32-8.34) remained stable and significantly lower.

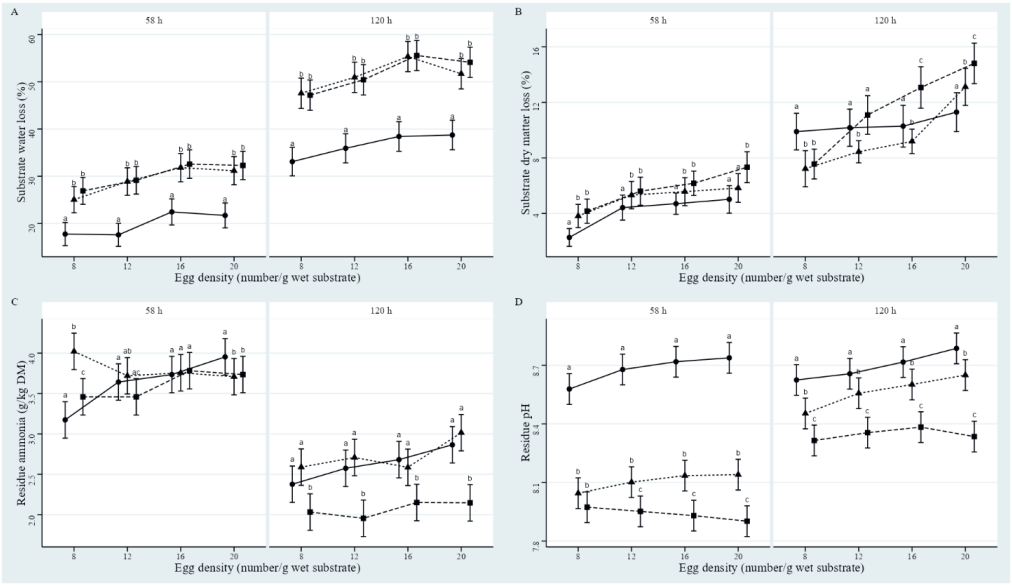


Figure 3. Substrate water loss (A), dry matter loss (B), residue ammonia content (C) and residue pH (D) of excreta substrates without addition (none, ●) or with glucose (▲) or starch (■), inoculated with different housefly egg densities and harvested at 58 h or 120 h. Dashes show the parameter value in fresh substrate. Symbols with whiskers show means and 95% confidence intervals. Means denoted by a different letter within egg density indicate significant differences between substrates. Data points were slightly offset along the x-axis to prevent overlap.

Factor analysis results

Table 3 shows the correlations and associated p-values between qualitative (substrate, ED) and quantitative variables and the first two dimensions obtained at 58 h and 120 h. The first dimension (36.1% explained variance) at 58 h, was primarily structured by biomass-related variables, showing strong correlations of BE_N, BE_{DM} and total larval wet weight ($r > 0.94$, $p < 0.001$), while substrate water and DM loss contributed moderately ($r = 0.74$, $p < 0.001$). Larval survival showed a weaker association ($r = 0.647$, $p < 0.001$) while residue pH, NH_3 and individual larval weight did not contribute significantly. Substrate was substantially more associated ($r = 0.728$, $p < 0.001$) than ED ($r = 0.132$, $p = 0.021$). The second 58 h dimension (16.1% explained variance) was defined almost entirely by individual larval wet weight ($r = 0.976$, $p < 0.001$). Residue NH_3 and BE_N were minor contributors (both $r = 0.260$, $p = 0.028$) while residue pH showed a significant negative correlation ($r = -0.331$, $p = 0.004$) . Egg density ($r = 0.770$, $p < 0.001$) was strongly associated to this dimension, whereas substrate was not ($r = 0.167$, $p = 0.002$). At 120 h, the first dimension (35.3% explained variance) was again associated with biomass-related variables, with strong correlations of total larval wet weight, BE_N and BE_{DM} and substrate water loss and residue NH3 also contributing notably. Interestingly, individual larval weight correlated negatively with this axis ($r = -0.606$, $p < 0.001$). Substrate was more strongly associated than at 58 h ($r = 0.266$, $p < 0.001$), while the association with ED remained strong ($r = 0.627$, $p < 0.001$). The second dimension at 120 h (17.4% explained variance)

showed a contrast between individual larval weight and substrate water loss (positive correlations) versus larval survival and substrate DM loss (negative correlations). Substrate was the dominant factor associated with this dimension ($r = 0.641$, $p < 0.001$) while ED had no significant influence ($r = 0.276$, $p = 0.062$).

Table 3. Correlations and associated p-values of quantitative and qualitative variables with the first two dimensions from a factor analysis of data from housefly larvae reared on chicken excreta without or with glucose or starch supplementation, harvested at 58 or 120 h.

	58 h		120 h	
Variable	**Dimension 1**	**Dimension 2**	**Dimension 1**	**Dimension 2**
	r / p	r / p	r / p	r / p
BE_N (%)[1]	0.951 / <0.001[2]	0.260 / 0.028	0.832 / <0.001	0.395 / 0.001
BE_{DM} (%)[2]	0.949 / <0.001	0.196 / 0.100	0.730 /<0.001	0.335 / 0.004
Total larval weight (g)	0.943 / <0.001	-0.214 / 0.072	0.893 / <0.001	-0.377 / 0.001
Individual larval weight (mg)	-0.061 / 0.612	0.976 / <0.001	-0.606 / <0.001	0.723 / <0.001
Survival (%)	0.647 / <0.001	-0.177 / 0.136	0.512 / <0.001	-0.568 / <0.001
Substrate water loss (%)	0.743 / <0.001	-0.057 / 0.632	0.694 / <0.001	0.521 / <0.001
Substrate dry mass loss (%)	0.724 / <0.001	-0.054 / 0.651	0.106 / 0.375	-0.704 / <0.001
Residue pH	0.029 / 0.807	-0.331 / 0.004	0.192 / 0.106	0.084 / 0.481
Residue ammonia (g/kg)	-0.054 / 0.650	0.260 / 0.028	0.631 / <0.001	0.338 / 0.004
Substrate type[3]	0.728 / <0.001	0.167 / 0.002	0.266 / <0.001	0.641 / <0.001
Egg density[3]	0.132 / 0.021	0.770 / <0.001	0.627 / <0.001	0.276 / 0.062

[1] Nitrogen bioconversion efficiency. [2] Dry matter bioconversion efficiency.[3] Qualitative variable.

Discussion

Optimizing larval survival and growth are the main drivers for efficient use of houseflies for manure bioconversion. Survival and growth can be both positively (improved digestion and decreased microbial competition) and negatively (competition for food and space) influenced by larval density, with positive effects more pronounced in younger larvae that benefit from higher densities. This study addressed the hypotheses that higher larval densities have higher survival than lower densities when starch is added to chicken excreta and that these effects are greater for younger larvae.

Effects of either glucose or starch supplementation and ED were indeed dependent on larval age at harvest (Figure 1C). For younger larvae (58 h harvest), survival in the GSE increased steadily with ED, surpassing both UE and SSE at higher ED. Survival in the SSE substantially increased only up to ED12 after which it fluctuated around 80%, similar to survival in the UE that showed no effect of ED. For older larvae, (120 h harvest), survival in the GSE increased strongly with ED and survival in the SSE now also showed substantial increase with ED, with both the GSE and SSE survival lower than that of the UE at low ED. The initial increase in survival of younger larvae in the GSE up to ED12 shows that larvae benefit from high larval density when starch is present up till a certain density. Then, additional larvae no longer enhance conditions for survival, possibly due to saturation of digestive enzyme activity or depletion of other nutrients that are needed. For older larvae, the continued increase in survival with higher ED when starch was added suggests benefits from

enhanced enzyme secretion or substrate modification. Amylase production may be age-dependent due to an ontogenetic dietary shift, or because young larvae cannot yet afford the associated high energetic and protein costs.

The effect of glucose compared to starch supports the temporal effects of starch. The differences in survival are in line with the bioavailability of both carbohydrates, with glucose being directly available for growth, while starch requires enzymatic digestion, potentially at the cost of energy. It could also be that glucose affected microbial or other characteristics of the substrate less negatively than starch during both growing periods, as indicated by the strong negative effect of starch on residue NH_3 content at the 120 h harvest, something that cannot be distinguished in this study. In the factor analysis, the 58 h survival showed a much stronger correlation with substrate than with ED (Table 3). Associations at the 120 h harvest were more complex, with survival more strongly correlated with ED than with substrate but also strongly negatively correlated with substrate dry matter loss and water loss. Differences in survival at 58 h were relatively small, but far larger at 120 h. Possibly, the large larval biomass at 58h generated by the addition of glucose and starch dried out the increasing mortality and decreasing larval biomass at 120 h (Figure 1B). This was not seen in UE that had substantially lower water loss and similar or higher larval biomass at the 120 h. Water loss was significantly higher in supplemented substrates at both harvest times. At 58 h, glucose- and starch-supplemented excreta lost around 25-32% water, compared to just 17-22% in UE. These trends remained at 120 h, with supplemented substrates losing more water over time (47 to 55% vs. 33 to 38%). Decreasing evaporation by increasing air humidity or adding water to substrates during the growing period to maintain a targeted moisture content could increase late harvest larval biomass.

Larval survival observed in the present study, especially for the GSE and UE at 58 h, showed unexpected variations with ED. This can be attributed to physical circumstances and propagated errors. During egg harvesting procedure the environment of the eggs changes as they are translocated from a milk-saturated cotton fabric to plastic weighing cups and then onto a crevice in the experimental substrates. Houseflies follow different chemical and biological clues for oviposition choosing specific micro-environments (Lam *et al*. 2007). The translocation of the eggs might have led to accidental drying of eggs or placement of eggs in an suboptimal micro-environment. A solution would be to have females oviposit “naturally” on substrates designed for young larvae and partition larvae into experimental substrates. This is common practice in rearing black soldier flies (Woods *et al*. 2019) but to the authors’ knowledge, this has not been described for the housefly. This, however, does not allow ED to be accurately fixed. Unexpected variation in survival estimates may also stem from error propagation in the calculation method. Both egg number (from egg mass) and larval count (from total larval mass and average individual weight) were estimated indirectly, allowing small inaccuracies to multiply and amplify overall variability. Studying survival would benefit greatly from a standardized procedure using a true egg count, inoculating small substrates with known numbers of eggs.

At the 58 h harvest, larval biomass, BE_{DM}, and BE_N were highly correlated into a mass-related metric together with survival and substrate but only weakly with ED (Table 3). This pattern reflects the

dominance of substrate quality over ED at the 58 h harvest. Both glucose and starch led to significant linear increases in larval biomass (glucose: +47%, starch: +49%), with associated but smaller gains in BE_{DM} (glucose: +12%, starch: +20%) and BE_N (glucose: +14%, starch: +21%). The slightly higher increases in BE_{DM} and BE_N with starch supplementation were primarily due to its somewhat broader range across ED, as BE_{DM} and BE_N showed minimal between-substrate differences (Figures 2A and 2B). At the 120 h harvest, survival, larval biomass, BE_{DM}, and BE_N remained aligned with the primary mass-related metric but their correlations were reduced (Table 3). Compared to 58 h, their association with ED became substantially stronger, while that with substrate substantially weakened. These changes indicate more complex interactions between ED and substrate, where water loss and possibly nutrient depletion by increased larval biomass differed between substrates. In SSE, larval biomass increased significantly only up to ED16 (+35%) while BE_{DM} and BE_N showed modest, non-significant increases (+8% and +10%, respectively), with asymptotic curves suggesting diminishing returns. In contrast, GSE again linearly increased larval biomass (+63%), while BE_{DM} (+47%) and BE_N (+46%) showed an asymptotic curve significantly surpassing SSE. These differences indicate that both glucose and starch supplementation using an ED of 20 eggs/g would maximize biomass and bioconversions for an early harvest (58 h). But, as was the case with survival, higher egg densities also accelerate substrate drying, potentially undermining long term larval survival and feeding efficiency. As larval biomass, survival and bioconversion are strongly related, time-sensitive management of substrates could remediate their long-term decrease, e.g. increasing air humidity or adding additional water during the growth period. Additionally, adding supplements, especially starch, not at the start but later might better maximize larval biomass and bioconversion.

Individual larva weight declined consistently with increasing ED for all substrates and both harvest times, clearly demonstrating density dependence. At 58 h, starch supplementation yielded the highest individual weights at low ED, but differences among substrates narrowed as ED increased, with differences becoming more pronounced at the 120 h harvest. Interestingly, factor analysis showed that individual larval weight belonged to an independent dimension of variation, separate from total biomass. The only notable correlation of individual mass at 58 h was with ED. This might indicate that individual mass is related to depletion of other nutrients than digestible carbohydrates. At 120 h, individual larva weight still was correlated an independent dimension (not related to total biomass), but its dominant correlations shifted to substrate and water loss, indicating the effect of substrates drying at 120 h. As individual larva weight is used here as the denominator in calculating survival, both dependence on unknown nutrients and heterogeneity of its distribution (individual weight tends to be more non-normal at higher larval density) might have confounded estimation of survival in this study. This again emphasizes the need for survival experiments with standardized egg numbers and nutritionally well-defined substrates.

Despite the strong effects of supplementation on larval biomass, BE_{DM} and BE_N at 58 h, DM losses were remarkably similar across substrates and only minimally affected by ED (Figure 3B). Still, DM loss attributed substantially to the mass-related dimension at this harvest time. This discrepancy indicates that both glucose and starch addition to chicken excreta increased the nitrogen efficiency

rather than the extent of DM degradation. For the 58 h growing period, the breakdown of substrate mass may still be limited as larvae primarily utilize more soluble nutrients (bacterial protein) that potentially only make up a small fraction of the total substrate mass. The 120 h substrate DM loss was substantially higher than the 58 h harvest and much stronger associated with ED in supplemented excreta, regardless of the lower total larval biomass. This suggests that older larvae affected the substrate more strongly. This is in line with the morphological changes in larval mouth parts occurring between the first and late instars. Compared to early instars, late instar mouth parts appear to allow for scraping, burying and ingestion of larger particles, indicating adaptation to different ingestible materials. As natural housefly larval substrates (e.g. small quantities of fresh feces or rotting fruit) are prone to drying out, the change in mouth parts might be an adaptation to a stickier or drier diet. Whereas there was no difference between supplements in relation to ED at 58 h, at 120 h SSE showed significantly higher substrate DM losses at higher ED. This suggests a timed density dependence, with starch utilization needing sufficient mass of older larvae and addition of starch to excreta only later in the larval growth period being optimal.

The NH_3 results are in line with the formation or accumulation of NH_3 as a time dependent process, associated with protein degradation or larval excretion over time. Glucose supplementation strongly increased early larval biomass, which may have led to more rapid, more larval N excretion and, therefore, greater NH_3 buildup by 120 h, particularly at higher ED. Surprisingly, NH_3 content of GSE and UE were highly similar at 120 h, with the first supporting much larger larval biomass at 58 h. This suggests that residue NH_3 content is not so much driven by larval excretion as by chemical or microbial characteristics of the substrate, that were less altered by glucose than by starch, affecting NH_3 formation, volatilization or retention. The lower NH_3 content of SSE residues supports the hypothesis that decreasing the dietary protein to energy ratio can lower NH_3 emission. A potential mechanism behind this could be the incorporation of N into bacterial biomass that is less prone to degradation into NH_3.

In contrast to NH_3, substrate residue pH was highly dependent on the substrate at 58 h, as indicated by its very low correlation with the mass-related dimension and weak effect of ED (Table 3, Figure 3D). As glucose and starch do not affect the pH directly, their observed association with pH must be due to microbial metabolism. Microbial production of acids, e.g. lactic acid or more volatile acids by species in genera such as *Lactobacillus* and *Enterococcus* is most common under anaerobic conditions (fermentation). This indicates that at least in the first 58 h, supplemented substrates were dense and sufficiently moist for this to occur. Surprisingly, residue pH did show a moderate negative correlation with individual larval mass, that was driven by ED (lower ED, heavier individuals, lower pH) in all substrates. The most likely explanation for this is that higher larval biomass suppressed increases in pH in GSE and SSE. At 120 h, the same pattern in residue pH occurred, with UE showing the highest, GSE lower and SSE the lowest pH. At this time point, GSE and UE were more similar and showed stronger effects of ED (higher ED, higher pH). As was the case for growth and survival, residue pH in the early stage of larval growth appears to be dependent mainly on substrate characteristics, whereas late stage pH is influenced mainly by biomass, particularly in GSE.

Conclusions

Carbohydrate supplementation of chicken excreta and larval density of house flies impact larval survival, biomass and substrate bioconversion in a time-dependent manner. At 58 h, both glucose and starch addition increase survival and biomass at higher egg density, with glucose showing stronger, more consistent effects. At 120 h, these effects weakened or reversed, likely due to increased substrate drying and possible nutrient depletion, particularly in glucose-supplemented substrates. Starch addition to excreta reduced NH_3 and pH of residues more strongly than glucose, suggesting differential microbial activity. Individual larval mass consistently declined with egg density and emerged as an independent dimension in factor analysis, suggesting nutrient limitations other than carbohydrate availability. Together, these results indicate that while carbohydrate supplementation and higher egg densities can enhance early-stage performance of house fly larvae, they also introduce risks for longer-term survival and bioconversion efficiency, especially through substrate drying.

References

Baldi, A., Gottardo, D. (2017). Livestock production to feed the planet. Animal Protein: A forecast of global demand over the next years. *Relations,* 5(1), 65-71.

Barragan-Fonseca, K. B., Dicke, M., van Loon, J. J. A. (2018). Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (*Hermetia illucens*). *Entomologia Experimentalis et Applicata,* 166(9), 761-770.

Bates, D., Mächler, M., Bolker, B., Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software,* 67(1), 1-48.

Boatta, F., Jansen, W. L., Beukeboom, L. W., Ellers, J. (2023). Lipid quantity and composition vary between three European house fly strains. *Entomologia Experimentalis et Applicata*, 171(4), 248-257.

Borchers, H. W. (2024). pracma: Practical Numerical Math Functions (Version 2.4.4) [R package]. https://CRAN.R-project.org/package=pracma

Britten, G. L., Dowd, M., Kanary, L., Worm, B. (2017). Extended fisheries recovery timelines in a changing environment. *Nature Communications*, 8, 15325.

Brookes, F. J., Frankel, G. (1958). The nutrition of the larva of the housefly. *Physiological Zoology,* 31(3), 208–223.

Canh, T. T. (1998). Ammonia emission from excreta of growing-finishing pigs as affected by dietary composition (Doctoral thesis). Wageningen Agricultural University, The Netherlands.

Cai, Y., Tang, R., Tian, L., Chang, S. X. (2021). Environmental impacts of livestock excreta under increasing livestock production and management considerations: Implications for developing countries. *Current Opinion in Environmental Science & Health,* 24, 100300.

Cai, Z., Yang, D., Wu, S., Wang, Y., Reaney, M. J. T., Zhao, Z., Zhu, L., Sun, G., Niu, Y., Zheng, D., Niu, H., Yang, W. (2017). Conversion of poultry manure to biodiesel, a practical method of producing fatty acid methyl esters via housefly (*Musca domestica* L.) larval lipid. *Fuel*, 210, 463-471.

Calvert, C. C., Morgan, N. O., Martin, R. D. (1970). House fly larvae: Biodegradation of hen excreta to useful products. *Poultry Science,* 49(2), 588-589.

Čičková, H., Pastor, B., Kozánek, M., Martínez-Sánchez, A., Rojo, S., Takáč, P. (2012). Biodegradation of pig manure by the housefly, *Musca domestica*: A viable ecological strategy for pig manure management. *PLoS ONE,* 7(3), e32798.

Čičková, H., Kozánek, M., Takáč, P. (2013). Improvement of survival of the house fly (*Musca domestica* L.) larvae under mass-rearing conditions. *Bulletin of Entomological Research,* 103(1), 119-125.

Cribari-Neto, F., Zeileis, A. (2010). Beta regression in R. *Journal of Statistical Software, 34*(2), 1-24.

FAO (Food and Agriculture Organization of the United Nations). (2011). *World Livestock 2011 - Livestock in Food Security*. Rome, Italy.

FAO (Food and Agriculture Organization of the United Nations). (2020). *The State of World Fisheries and Aquaculture: Sustainability in Action.*

Fawole, F. J., Shamna, N., Memudu, H. A., Abdullahi, N., Hassaan, M. S., Gbadamosi, O. K. (2023). Housefly maggot meal complement soybean meal in a fish-free diet for hybrid catfish (*Clarias gariepinus* ♀ x *Heterobranchus longifilis* ♂): Effect on growth, body composition, blood biochemistry and antioxidant enzyme activity. *Animal Feed Science and Technology,* 295, 115543.

Gadzama, I. U., Ndudim, R. K. (2019). Nutritional composition of housefly larvae meal: A sustainable protein source for animal production - a review. *Acta Scientific Agriculture*, 3, 74-77.

Gerberich, J. B. (1948). Rearing house-flies on common bacteriological media. *Journal of Economic Entomology,* 41(1), 125-126.

Greenspoon, L., Krieger, E., Sender, R., Rosenberg, Y., Bar-On, Y. M., Moran, U., Antman, T., Meiri, S., Roll, U., Noor, E., Milo, R. (2023). The global biomass of wild mammals. *Proceedings of the National Academy of Sciences*, 120(10), e2204892120.

Grossule, V. (2024). Wastewater treatment using black soldier fly larvae: Influence of larval density and solid support. *Journal of Water Process Engineering*, 67, 106214.

Heuel, M., Sandrock, C., Leiber, F., Mathys, A., Gold, M., Zurbrügg, C., Gangnat, I. D. M., Kreuzer, M., Terranova, M. (2021). Black soldier fly larvae meal and fat can completely replace soybean cake and oil in diets for laying hens. *Poultry Science*, 100(4), 101034.

Hirashima, A., Oyama, K., Ueno, R., Ishaaya, I., Eto, M. (1989). Carbohydrate enzymes in house fly *Musca domestica* larvae: Biochemical properties and toxicological significance. *Journal of the Faculty of Agriculture, Kyushu University,* 34(1/2), 37-42.

Huang, D., Gao, L., Cheng, M., Yan, M., Zhang, G., Chen, S., Du, L., Wang, G., Li, R., Tao, J., Zhou, W., Yin, L. (2022). Carbon and N conservation during composting: A review. *Science of the Total Environment*, 840, 156355.

Hussein, M., Pillai, V. V., Goddard, J. M., Park, H. G., Kothapalli, K. S., Ross, D. A., Selvaraj, V. (2017). Sustainable production of housefly (*Musca domestica*) larvae as a protein-rich feed ingredient by utilizing cattle manure. *PLOS ONE*, 12(2), e0171708.

Khoshnevisan, B., Duan, N., Tsapekos, P., Awasthi, M. K., Liu, Z., Mohammadi, A., Angelidaki, I., Tsang, D. C., Zhang, Z., Pan, J., Ma, L., Aghbashlo, M., Tabatabaei, M., Liu, H. (2020). A critical review on livestock manure biorefinery technologies: Sustainability, challenges, and future perspectives. *Renewable and Sustainable Energy Reviews*, 135, 110033.

Kortsmit, Y., van der Bruggen, M., Wertheim, B., Dicke, M., Beukeboom, L. W., van Loon, J. J. A. (2023). Behaviour of two fly species reared for livestock feed: Optimising production and insect welfare. *Journal of Insects as Food and Feed*, 9(2), 149-170.

Lam, K., Babor, D., Duthie, B., Babor, E.-M., Moore, M., Gries, G. (2007). Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. *Animal Behaviour*, 74(1), 81-92.

Lam, K., Thu, K., Tsang, M., Moore, M., Gries, G. (2009). Bacteria on housefly eggs, *Musca domestica*, suppress fungal growth in chicken manure through nutrient depletion or antifungal metabolites. *Naturwissenschaften*, 96, 1127-1132.

Lê, S., Josse, J., Husson, F. (2008). FactoMineR: A Package for Multivariate Analysis. *Journal of Statistical Software*, 25(1), 1-18.

Levinson, Z. H. (1960). Food of housefly larvae. *Nature*, 188, 427-428.

McCommas, S., Pryde Shornick, L. (1990). The effect of carbohydrate sources on the level of amylase activity in *Musca domestica*. *Biochemical Genetics*, 28(11/12), 585-589.

Miller, B. F., Teotia, J. S., Thatcher, T. O. (1974). Digestion of poultry manure by *Musca domestica*. *British Poultry Science*, 15(2), 231-234.

Miranda, C. D., Cammack, J. A., Tomberlin, J. K. (2020). Life-history traits of house fly, *Musca domestica* L. (Diptera: Muscidae), reared on three manure types. *Journal of Insects as Food and Feed*, 6(1), 81-90.

Miranda, C. D., Cammack, J. A., Tomberlin, J. K. (2023). Large-scale production of house fly, *Musca domestica* (Diptera: Muscidae), larvae fed 3 manure types. *Journal of Economic Entomology*, 116(4), 1102-1109.

Moon, R. D., Hinton, J. L., O'Rourke, S. D., Schmidt, D. R. (2001). Nutritional value of fresh and composted poultry manure for house fly (Diptera: Muscidae) larvae. *Journal of Economic Entomology*, 94(5), 1308-1317.

Mottet, A., de Haan, C., Falcucci, A., Tempio, G., Opio, C., Gerber, P. (2017). Livestock: On our plates or eating at our table? A new analysis of the feed/food debate. *Global Food Security*, 14, 1-8.

Nayduch, D., Burrus, R. G. (2017). Flourishing in filth: House fly–microbe interactions across life history. *Annals of the Entomological Society of America*, 110(1), 6-18.

Newton, L., Sheppard, C., Watson, D., Burtle, G., Dove, R. (2005). Using the black soldier fly, *Hermetia illucens*, as a value-added tool for the management of swine manure. Raleigh (NC): Animal and Poultry Waste Management Centre, North Carolina State University.

Opare, L. O., Holm, S., Esperk, T. (2022). Temperature-modified density effects in the black soldier fly: Low larval density leads to large size, short development time and high fat content. *Journal of Insects as Food and Feed*, 8(7), 783-802.

Parodi, A., Gerrits, W. J. J., van Loon, J. J. A., de Boer, I. J. M., Aarnink, A. J. A., van Zanten, H. H. E. (2021). Black soldier fly reared on pig manure: Bioconversion efficiencies, nutrients in the residual material, greenhouse gas and ammonia emissions. *Waste Management*, 126, 674-683.

Pimentel A.C., Barroso I.C., Ferreira J.M.J., Dias R.O., Ferreira C., Terra W.R. (2018). Molecular machinery of starch digestion and glucose absorption along the midgut of *Musca domestica*. *Journal of Insect Physiology*, 109, 11-20.

Pisa, L. W., Oonincx, D. G. A. B., Bosch, G., Hendriks, W. H. (2022). Bioconversion of fresh chicken excreta by housefly larvae (*Musca domestica* L.) in relation to excreta sterilisation and carbohydrate addition. *Journal of Insects as Food and Feed, 8*(10), 1107-1116.

Pisa, L. W., Oonincx, D. G. A. B., Bosch, G., Hendriks, W. H. (2024). Dose-response effects of starch in sterilised and unsterilised chicken excreta on Musca domestica larval growth and bioconversion efficiency. *Journal of Insects as Food and Feed*, 1-12.

Pieterse, E. and Pretorius, Q. (2014). Nutritional evaluation of dried larvae and pupae meal of the housefly (*Musca domestica*) using chemical and broiler based biological assays. *Animal Production Science*, 54(3), 347-355.

Pieterse, E., Pretorius, Q., Hoffman, L. C. (2014). The carcass and meat quality of the black soldier fly (*Hermetia illucens* L.) larvae fed on different substrates. *Animal Production Science,* 54(3), 347-355.

Prăvălie, R., Patriche, C., Borrelli, P., Panagos, P., Roșca, B., Dumitrașcu, M., Nita, I.-A., Săvulescu, I., Birsan, M.-V., Bandoc, G. (2021). Arable lands under the pressure of multiple land degradation processes. A global perspective. *Environmental Research*, 194, 110697.

R Core Team. (2023). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Rama, T.K.E., Tongol, M.A.S. (2024). Digestibility of different carbohydrate sources in the diet of native chicken (*Gallus gallus domesticus*). *International Research Journal of Science, Technology, Education and Management*, 4(2), 158-166.

Sánchez, M., Gómez, C., Avendaño, C., Harmsen, I., Ortiz, D., Ceballos, R., Villamizar-Sarmiento, M. G., Oyarzun-Ampuero, F., Wacyk, J., Valenzuela, C. (2021). House fly (*Musca domestica*) larvae meal as an ingredient with high nutritional value: Microencapsulation and improvement of organoleptic characteristics. *Food Research International,* 145, 110423.

Scanvion, Q., Hédouin, V., Charabidzé, D. (2018). Collective exodigestion favours blow fly colonization and development on fresh carcasses. *Animal Behaviour,* 141, 221-232.

Snow, G. (2020). blockrand: Randomization for block random clinical trials. *R package version 1.5*.

Svihus, B. (2014). Starch digestion capacity of poultry. Poultry Science, 93(9), 2394-2399.

Szpila, K., Pape, T. (2008). Morphology of the first instar of the house fly *Musca domestica* (Diptera: Muscidae). *Journal of Medical Entomology,* 45(4), 594-599.

Tomberlin, J. K., Adler, P. H., Myers, H. M. (2009). Development of the black soldier fly (Diptera: Stratiomyidae) in relation to temperature. *Environmental Entomology,* 38, 930-934.

van Zanten, H. H. E., Mollenhorst, H., Oonincx, D. G. A. B., Bikker, P., Meerburg, B. G., de Boer, I. J. M. (2015). From environmental nuisance to environmental opportunity: housefly larvae convert waste to livestock feed. *Journal of Cleaner*

Weihrauch, D., O'Donnell, M. J. (2021). Mechanisms of nitrogen excretion in insects. *Current Opinion in Insect Science,* 47, 25-30.

Woods, M. J., Hoffman, L. C., Pieterse, E. (2019). Artificial diets for neonatal black soldier fly (*Hermetia illucens*) larvae. *Journal of Insects as Food and Feed*, 5(2), 99-106.

Wu, L., Zhang, X., Tang, Z., Li, Y., Li, T., Xu, Q., Zhen, J., Huang, F., Yang, J., Chen, C., Wu, Z., Li, M., Sun, J., Chen, J., An, R., Zhao, S., Jiang, Q., Zhu, W., Yin, Y., Sun, Z. (2018). Low-protein diets decrease porcine nitrogen excretion but with restrictive effects on amino acid utilization. *Journal of Agricultural and Food Chemistry*, 66(31), 8262-8271.

Production, 102, 362-369.

Supplementary material.

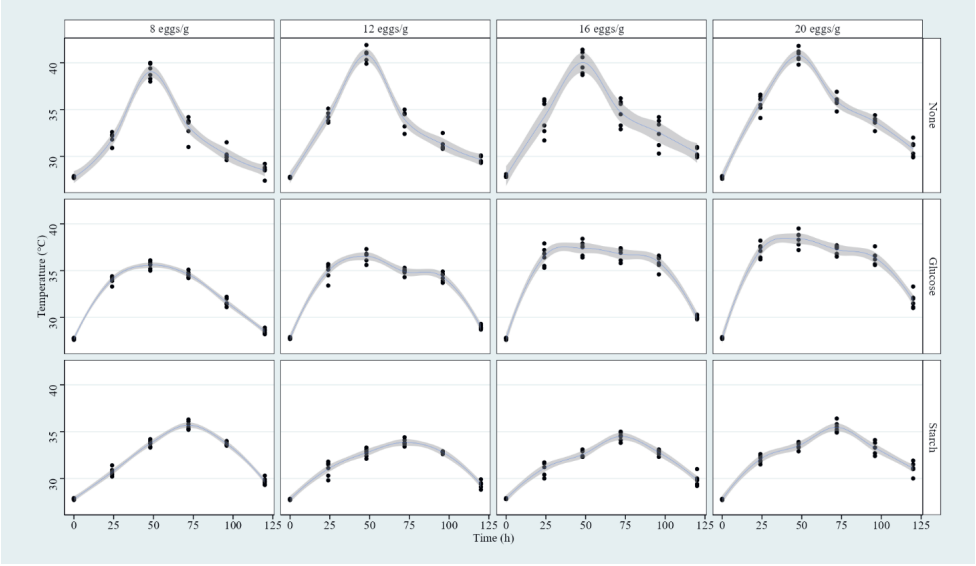


Figure S1. Temperature profiles of chicken excreta without (none) or with glucose or starch addition, harvested at 120 h and inoculated with different housefly egg densities. Shaded areas represent 95% confidence intervals.

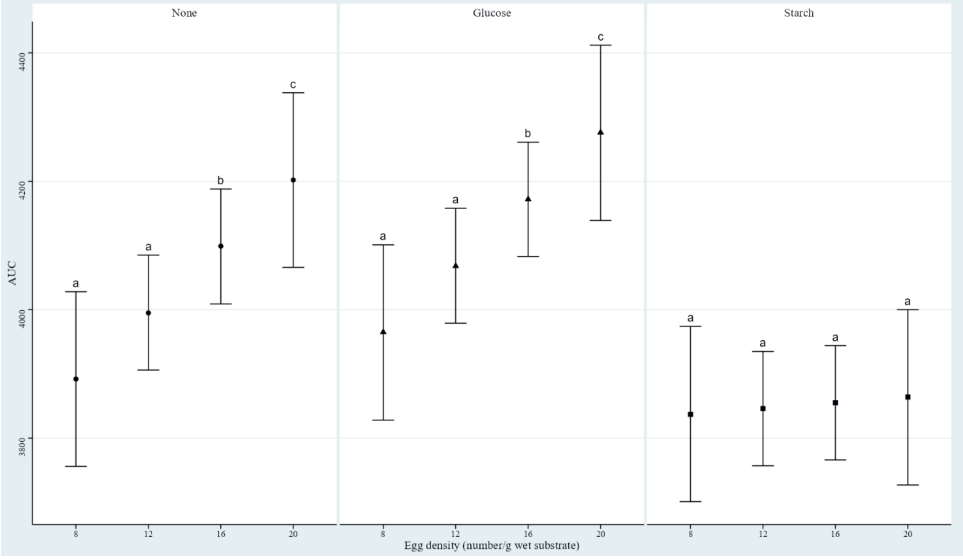


Figure S2. Total heat produced (AUC, area under the curve) of chicken excreta without (none) or with glucose or starch addition and inoculated with different housefly egg densities. Symbols with whiskers show means and 95% confidence intervals. Means denoted by a different letter indicate significant differences between egg densities within substrate type

Chapter 6

General discussion

6.1 Introduction

Animal manures can represent a valuable resource for nutrient recycling. In addition, the mass-rearing of housefly larvae (*Musca domestica*) offers a promising approach for generation of protein-rich biomass that is highly suitable for animal feed. Larval nutritional requirements are relatively well known from studies using (semi)-synthetic substrates. For animal manure, with its complex and changing material (see **Chapter 1**), to be optimized for larval growth, substantially more knowledge of larval nutrient utilization and microbial ecology is required.

This thesis examined how chicken excreta can be optimized as a substrate to enhance housefly larval growth and the bioconversion of dry matter (DM) and nitrogen (N), with particular emphasis on digestible carbohydrates, especially starch. Housefly larvae can use starch as an energy source (see **Chapter 1**), hence supplementing protein-rich excreta with starch may increase larval biomass and bioconversion efficiency of DM and N by enhancing individual weight and/or survival through more efficient utilization of dietary protein. A more efficient utilization can be achieved through two mechanisms. First, starch provides energy allowing dietary protein to be directed toward amino acid deposition and tissue growth rather than oxidation. Secondly, reduced protein catabolism lowers nitrogenous waste (primarily uric acid), decreasing the uric acid available for microbial conversion into ammonia and subsequent volatilization. However, starch may also be used by native and non-native (airborne) excreta microorganisms, potentially creating competition that could adversely affects larval survival and growth (see **Chapter 1**).

Hitherto, no prior knowledge existed on how starch supplementation of excreta influences larval growth and substrate bioconversion, interacting with microbial load, larval developmental stage and larval density. A series of experiments was conducted to address these gaps, with their rationale and framework outlined in **Chapter 1**. In **Chapter 2**, the effects of microbial activity on larval growth and bioconversion were examined through heat sterilization of starch-supplemented excreta. **Chapter 3** investigated the dose-response relationship of starch addition to identify concentrations that maximize larval growth and bioconversion. **Chapter 4**, evaluates the timing of starch addition to investigate stage-specific starch utilization, contrasting supplementation at the start and later in development. Finally, **Chapter 5** explored the role of larval density, testing whether higher densities can counteract the reduced survival observed with starch addition.

6.2 Excreta sterilization, starch addition and larval growth

Heat sterilization (**Chapters 2** and **3**) increased excreta DM and total N content while reducing uric acid and ammonia levels. Ammonia likely volatilized during heating, whereas the uric acid decline may reflect enhanced oxidation or uricase activity during the heating process. Individual larval weight and larval biomass were consistently 5-15% lower in sterilized excreta compared to unsterilized excreta, possibly due to heat-sensitive nutrient losses (e.g. B vitamins) and/or the reduction of specific microbial activity that supports larval growth. The latter can consist of a lack of microbial enzyme secretions resulting in reduced digestibility of the substrate or reduced availability of microbial biomass.

Proof of principle

In the **Chapter 2** experiment, the combination of starch addition (18% substrate DM) and heat sterilization increased individual and total larval biomass. Compared to unsterilized starchless excreta, total larval biomass was lower in sterilized starchless excreta (-18%), significantly higher in sterilized excreta with starch (35%) and much lower in unsterilized excreta with starch (-54%). These results support the hypotheses that housefly larvae can use starch when added to excreta and compete with microorganisms for starch. Although these hypotheses are supported, it is unclear if this reflects true nutritional competition for starch or a non-nutritional effect, such as production of microbial toxins or alterations in essential microbial presence.

Considering total larval biomass as the product of survival and individual larval weight reveals to some extent how starch addition and sterilization interacted. Sterilized starchless excreta maintained a high survival (both ~70%), combined with a moderate reduction in mean individual weights and a moderate decrease in biomass compared to unsterilized starchless excreta. These findings indicate that individual growth is the dominant factor driving larval biomass in sterilized starchless substrate. In contrast, unsterilized excreta with starch (18% DM) showed a large reduction in survival (-51%), a slight decrease of individual larval weight and a large reduction of total biomass. This points towards survival as the dominant driver of total biomass in the presence of both starch and living excreta microorganisms. Starch addition to sterilized excreta moderately reduced survival (-19%) while strongly increasing individual weight and total biomass. So in this case, individual growth was the dominant driver of total biomass and compensated reduced survival.

Substrate microbiology and larval growth, clues from chemical parameters

Changes in uric acid, ammonia, pH, and C/N between fresh larval substrates and substrate residues found in **Chapter 2** and **3** provide insights into the possible microbial activity underlying the differences in biomass and survival.

In unsterilized starchless excreta (high survival, moderately heavy larvae, moderate total biomass), uric acid degradation was extensive (92%) while a high residue ammonia content (0.4% DM) was combined with an increase in pH (7.1 to 8.2). The C/N increased (9 to 16), pointing to N loss through volatilization of ammonia. These changes indicate activity of ureolytic bacteria such as *Proteus*, *Morganella* or *Klebsiella* spp., that are common in poultry manure and part of the housefly egg microbial symbionts (**Chapter 1**, Box 2). These bacteria hydrolyze uric acid and urea into ammonia that elevates substrate pH. The resulting alkaline conditions suppress microbial competitors such as fungi and potentially improve protein availability. At pH 8-9, many proteins undergo partial denaturation that exposes peptide bonds and increases their susceptibility to hydrolysis (Hadinoto *et al*. 2024). This is particularly advantageous for larvae because larval proteases exhibit optimal activity in the pH 8-9 range, matching conditions in the posterior midgut (Blahovec *et al*. 2006). Ureolytic bacteria are deposited with housefly eggs and their rapid, local, effects might be crucial for survival of the youngest larvae that depend on the abundant bacterial protein in fresh excreta (as proposed in **Chapter 1**).

Compared to unsterilized, sterilized starchless excreta (characterized by high survival of moderately heavy larvae and a moderate biomass yield) showed significantly lower uric acid degradation (45%), while ammonia (0.4% DM) and pH (~8.5) remained similar. The C/N increased less after sterilization (+3 vs. +7), indicating reduced N loss. These findings indicate that ureolytic bacteria from housefly eggs or the non-sterile environment enhanced ureolytic activity despite sterilization, maintaining alkaline conditions that supported larval survival. The lower individual and total biomass likely reflected the sterilization-related breakdown of essential nutrients or microorganisms. Adding 18% starch to unsterilized excreta disrupted the ureolytic environment, resulting in low larval survival and biomass. Substrate residues contained no detectable ammonia, slightly higher uric acid levels (+2% compared to the fresh substrate) and showed a strong decrease in pH (7.1 to 5.3) and C/N (-7), indicating substantial C loss relative to nitrogen. These changes are consistent with the growth of lactic acid producing bacteria (*Lactobacillus* and *Enterococcus* spp.) that ferment starch to lactic acid, with other species (Bacillus and Clostridium spp.) and the extracellular amylase producing fungi (*Mucor*, *Aspergillus* spp.) metabolizing starch to carbon dioxide in neutral to acidic conditions (**Chapter 1**). Apart from direct competition effects, the amylolytic-acidic conditions might have inhibited (early) larval protein utilization which requires alkaline conditions.

Compared to unsterilized excreta, the combination of sterilization and starch significantly increased larval weight and total biomass but decreased survival. Substrate residues contained low ammonia (0.1% DM), slightly higher uric acid levels (+2%), and a low pH (4.6). The C/N decreased (-5), indicating C loss, likely driven by starch utilization and microbial respiration under limited ureolytic activity. Rapid ureolytic activity near the eggs may have created brief alkaline conditions essential for early larval survival, preventing the decline observed in unsterilized starch-supplemented excreta.

In the **Chapter 2** experiment, fructo-oligosaccharides (FOS) were also added to sterilized or unsterilized chicken excreta. Commonly used as prebiotics in mammalian nutrition, FOS are fermented by gut bacteria such as *Bifidobacterium* spp., producing volatile fatty acids and result in changes in microbial abundance that benefit gut health (Stefanelli *et al*. 2016, Dou *et al*. 2022). Unlike starch, FOS can only be metabolized by bacteria and not by larvae. Thus, in theory, FOS addition to unsterilized excreta could have enhanced larval biomass by stimulating specific bacterial growth beneficial for larvae. However, FOS caused over 90% mortality in its substrates, leading to minimal larval biomass. This effect was likely caused by a pronounced increase in substrate viscosity, turning excreta into syrupy mass, inhibiting larvae from burying into the substrate. Based on this limited experiment, FOS used at 18% substrate DM appears of no use to enhance larval growth in excreta. But testing lower FOS concentrations could help determine whether moderate prebiotic stimulation of microbial activity benefits larval performance without compromising substrate structure.

Starch-induced microbial thresholds and larval growth

Chapter 3 provided a more nuanced view on the relationship between larval growth, sterilization and starch addition. Adding 0 to 50% starch (of substrate DM) to unsterilized and sterilized excreta revealed that the interaction between starch addition and sterilization produced pronounced threshold effects.

In **Chapter 3**, mean individual larval weight peaked at 15% starch (~18 mg). Relative to 0% starch, weight increased more in unsterilized (+52%) than in sterilized excreta (+40%). Beyond 15% starch, individual weight declined sharply in unsterilized excreta but more gradually in sterilized that maintained near-peak weights up to 20% starch. Mean total larval biomass peaked at 10% starch in unsterilized and at 15% starch in sterilized excreta, representing increases of 21% and 37%, respectively, relative to starchless excreta. After these maxima, biomass declined rapidly in unsterilized excreta (<10% of peak by 35% starch) and more slowly in sterilized excreta. Survival decreased almost linearly with increasing starch concentration in unsterilized (80 to 14%) and in sterilized excreta (72 to 18%).

To more closely examine the relationship between larval biomass, residue pH, ammonia and uric acid in response to starch addition and sterilization, these **Chapter 3** variables were rescaled using min-max normalization to enable comparison of relative patterns across their different units (Figure 1).

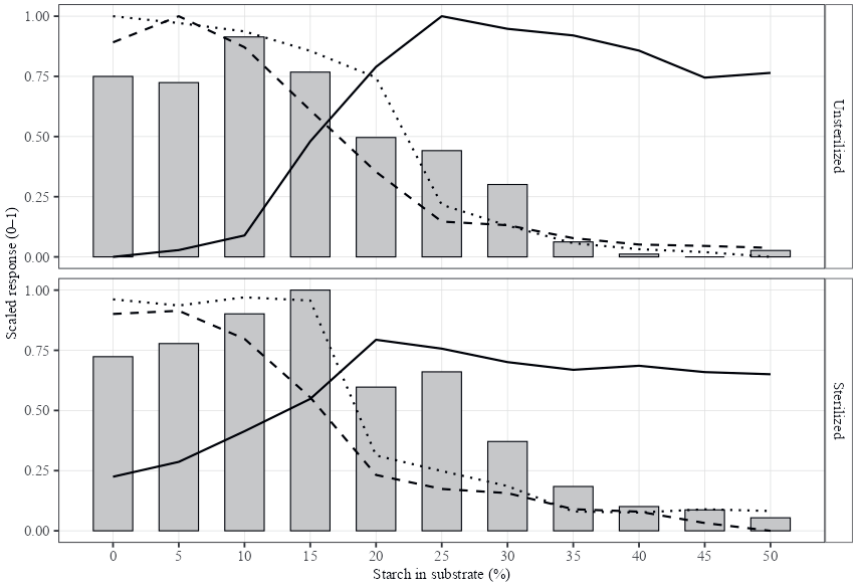


Figure 1. Scaled responses of uric acid recovered (—), residue ammonia (- - -) and residue pH (···) in relation to larval biomass (grey bars) across starch concentrations in unsterilized and sterilized excreta substrates. Each variable was rescaled to a 0-1 range using min-max normalization.

In unsterilized excreta, larval biomass peaked at 10% starch and declined beyond 15%, coinciding with a shift from alkaline, ureolytic conditions (high pH and ammonia) to acidic conditions with lower uric acid breakdown. In sterilized excreta, these transitions occurred slightly later and a sharper decrease was observed after the peak biomass at 15% starch indicating a greater tolerance to starch enrichment. The decoupling of pH and ammonia marked the breakdown of ureolytic activity, aligning with biomass peaks in both substrates. Starch recovery increased with starch addition, stabilizing around 17% in unsterilized and 23% in sterilized excreta, indicating lower microbial conversion at

higher starch levels. Together, these patterns indicate that larval performance depends on maintaining a ureolytic, ammonia-buffered environment and that excess starch drives a chemical shift toward acidic conditions incompatible with larval growth.

The nutritional ecology framework constructed in **Chapter 1** allows for speculation on the mechanisms behind these patterns. In unsterilized excreta, the 10% starch concentration appeared to shift microbial diversity and abundance. Below this concentration, ureolytic bacteria dominate, producing ammonia that sustain alkalinity and suppress starch-utilizing microorganisms. At higher concentrations, acidogenic microorganisms gain dominance over ureolytic ones, lowering pH sufficiently to inhibit enzyme-driven ammonia generation. This shift coincides with peak larval biomass at 10%, followed by a strong decline at higher starch concentrations. Sterilization displaces this threshold to a higher starch concentration, with the transition from alkaline to acidic conditions occurring at ~15-20%.

While absolute changes in ammonia content and pH were broadly comparable between sterilized and unsterilized excreta up to 25% starch, the relationship between these parameters diverged earlier in sterilized excreta where the pH was maintained at higher alkaline levels while ammonia declined. In parallel, uric acid degradation was much lower up to 15% starch addition, corresponding to the threshold for peak larval biomass in sterilized excreta. Starch recovery also remained lower up to 25% starch, suggesting increased uptake by larvae and/or a restricted set of beneficial microorganisms. As shown in **Chapter 2**, larval starch uptake primarily drove increased individual growth, which in turn determined higher total biomass despite starch also reducing survival. The delayed shift to acidogenic dominance in sterilized excreta, therefore, extended the concentration up to which larvae could utilize starch without major microbial competition or substrate acidification.

Direct uptake and digestion of starch by larvae are highly likely (**Chapter 1**) but alternative pathways of larval starch use cannot be excluded. Small amounts of starch may stimulate specific microbial groups that provide (additional) microbial biomass as food. More exotic routes, such as starch hydrolysis to glucose by gut enzymes present in the excreta followed by subsequent larval uptake of glucose also remain possible.

As in the research described in this thesis no microorganisms were identified, analogies with other systems can provide extra clues on the microbiota involved in starch-induced microbial shifts. In pigs, raising resistant starch above 15-25% of the diet shifts large intestinal fermentation from proteolytic taxa (*Clostridium* spp., Bacteroides) to acid-tolerant amylolytic fermenters (*Lactobacillus*, *Bifidobacterium*, *Streptococcus* spp.), lowering pH and increasing lactic acid and acetate production (Tan *et al*. 2014, Metzler-Zebeli *et al*. 2017). Similar patterns occur in chicken ceca, where starch promotes *Lactobacillus* and *Bifidobacterium*, reduces *Clostridium perfringens* while decreasing pH from ~6.5 to ~5.5 (Zhang *et al*. 2020), and in humans and rats, where 10-20% resistant starch shifts dominance toward lactic acid producers reducing ammonia and proteolytic byproducts (Morita *et al*. 1998, Topping and Clifton 2001). In each case, once carbohydrate supply surpasses proteolytic capacity, amylolytic fermenters dominate, acidifying the environment and changing relative species

abundance. However, the species associated with (moderate) alkaline conditions in animal guts are different taxa (*Clostridium* spp., *Bacteroides*) than the symbiotic "*Proteus* group" larval symbionts (*Proteus*, *Klebsiella*, *Pseudomonas* spp.) identified in **Chapter 1**. The proteolytic Clostridiales and Bacteroidetes are predominantly anaerobic and generate ammonia and weak bases such as isobutyrate by deaminating amino acids. In contrast, the ureolytic species of the "*Proteus* group" are facultative anaerobes that obtain both nitrogen and C from uric acid or urea. If uric acid or urea are present, uricases and ureases of the *Proteus* group generate ammonia much faster than *Clostridium* spp. and Bacteroidetes (Mobley *et al*. 1995, Tiquia and Tam 2000). The resulting speed of alkalinization might be essential for very young larvae for reasons mentioned earlier. Most laboratory larval substrates are bran based and so contain at least 10-20% starch (**Chapter 1**). Several studies have specified the microorganisms in these substrates and in the larval gut and either *Proteus* or the similar *Providencia* spp. were always present (de Jonge *et al*. 2020, Zhao *et al*. 2017, Zurek *et al*. 2000).

I consider substrate odor an important indicator of larval growth, smelling odor contrasts in hundreds of culture and experimental substrates (**Appendix 2**). Though subjective, odor observations from **Chapter 3** can be considered chemical cues that match the proposed microbial transitions. In unsterilized substrates with ≤10% starch, strong ammonia odor indicated ureolytic activity by the *Proteus* group. At >15-20% starch, the odor transitions to "fruity", reminiscent of cheap strawberry candy. This suggests the presence of lactic acid bacteria and yeasts that produce volatile esters (such as ethyl acetate that has a banana like odor) and alcohols during carbohydrate fermentation (Smid and Kleerebezem 2014). In sterilized substrates, ammonia odor persisted up to 15% while beyond 20% starch, the odor shifted to "woody", best described as a mixture of fresh wood shavings and disinfectant. This could be related to the production of volatile organic compounds, such as 1-octen-3-ol and 1-octen-3-one by filamentous fungi, e.g. *Aspergillus* or *Mucor* spp. (Morath *et al*. 2012, Poulaki and Tjamos 2023).

The importance of suppressing fungi (**Chapter 1**) is demonstrated by the common use of methylparaben (methyl p-hydroxybenzoate) in bran-based housefly substrates. Methylparaben is a widely used preservative that disrupts microbial cell membranes, with fungi being particularly susceptible (Freese *et al*. 1973, Kwan *et al*. 2014). It is also commonly added to *Drosophila* substrates, that are mostly bran- or cornmeal-based with added yeast and a sugar source (glucose, sucrose). I only observed fungi in sterilized excreta with >40% starch and overly wet wheat bran substrates and found that methylparaben in bran-containing housefly substrates can be omitted when inoculated with (very) high densities of larvae, indicating an inhibitory effect of larvae and/or their symbiotic bacteria (**Appendix 2**).

Klammsteiner *et al*. (2025) applied potassium sorbate, primarily a fungicide, to food waste substrates for BSF larvae and observed shifts in microbial abundance and biochemical parameters. Their fresh food waste (~3% N, C/N ~17, pH ~5.4) differs substantially from the starch-supplemented excreta that was used in the research described in this thesis (~5% N, C/N ~10, pH ~7.8). In the absence of larvae, food waste acidified from pH 5.4 to 3.6-3.9, indicating activity of acidifying bacteria, but high larval densities resulted in alkaline substrate residues (pH 7.8-7.9), similar to what was seen for

housefly larvae (**Chapter 5**). Potassium sorbate suppressed fungi and reduced abundance of *Providencia* and *Actinomyces*, while enriching *Enterobacter* spp. Sorbate reduced peak larval biomass at medium density and slowed larval growth at high density. From a housefly perspective, the reduction in *Providencia*, a member of the ureolytic *Proteus* group (**Chapter 1**, Box 2) might have reduced alkalinity that is thought to be beneficial for early larval nutrition and survival. Interestingly, Klammsteiner *et al*. (2025) proposed that sorbate mainly slowed larval growth because young larvae depend on microbial substrate degradation for nutrient uptake. They could not support this hypothesis with stage specific survival data nor did they specify a mechanism for it.

6.3 Timing of starch addition

Larval survival is a key determinant of total biomass. **Chapter 1** suggests that the larval diet changes with larval development, based on differential morphology of mouth parts and presence of proteases and amylases. Young larvae could be mainly dependent on soluble protein while older larvae ingest and digest complete bacteria. **Chapter 2** and **3** showed that starch addition reduced larval survival and altered chemical parameters (pH, ammonia) that potentially affected young larvae the most, leading to low early survival. **Chapter 4** explores the idea that the timing of starch addition affects larval growth and survival, testing the hypothesis that later addition during the larval growth period results in higher survival and growth than addition at start. Starch, glucose or cellulose were mixed into excreta as 10% of total DM, either early (0 h, before inoculation with eggs) or late (72 h). Glucose was included as a positive control to assess whether larval growth is limited solely by the availability of a digestible carbohydrate. Because glucose can be utilized by all larval instars without prior digestion, its effects were expected to match those of starch if larval starch utilization is not stage-dependent. Cellulose, indigestible for larvae, served as a negative control to account for any effects of physically mixing materials into the excreta during larval growth.

Glucose addition produced the heaviest larvae and the largest increase from early to late supplementation, with mean individual weight and mean total biomass increasing by 12% and 33%, respectively. Starch addition yielded smaller gains, with individual weight increasing 4% and total biomass by 29% from early to late addition, whereas cellulose and excreta showed negligible or negative effects on individual weight and total biomass. Compared with early excreta addition, total biomass increased 70% with late glucose and 30% with late starch, while early starch provided little benefit (+1%). Survival increased by 19% with early glucose and decreased by 3% with early starch, while increasing 2 to 3% with early and late cellulose and decreasing 9% with late excreta. The negative effect of early starch combined with the positive effects of late starch and both early and late glucose support the hypothesis that survival is more sensitive to the timing of starch addition than to glucose addition. Considering total biomass, these results indicate that growth in excreta was indeed limited by the lack of a digestible carbohydrate and that the biomass gains from starch addition were mainly driven by improved survival rather than increased individual growth.

Ammonia and pH of substrate residues showed clear contrasts between glucose and starch additions. Residual ammonia concentrations were consistently high with glucose addition timing (-2% difference) while late starch addition was associated with a marked reduction (-11% difference). Early

to late glucose residues showed a slight decrease in pH (-1%), whereas early to late starch residue pH increased slightly (+3%).

This decoupling of residue ammonia content and pH was seen before in **Chapter 3**, where it started at 10% starch in unsterilized excreta and coincided with maximal total biomass (Figure 1 in section 6.1). Here, the decoupling was specific to late starch addition, as early starch showed the expected alignment of lower ammonia with lower pH. A possible explanation is that with late starch addition, compared to glucose and early starch, larval emission of uric acid available for degradation to ammonia was reduced because older larvae were better able to utilize starch as an energy source. In addition, microbial activity may have been time dependent, with specific species assimilating more ammonia into microbial biomass while not yet producing enough acidic metabolites to counteract alkalinity.

6.4 Starch addition, larval density and larval survival

As seen in **Chapter 2**, **3** and **4**, starch addition can increase larval biomass in unsterilized excreta but it also decreases larval survival. Adding starch later in the larval development mitigated this effect, leading to higher survival and total biomass. However, adding starch to substrates later during larval growth might be operationally more complicated than incorporating starch during substrate preparation. An alternative approach to increase survival in starch containing excreta is using higher larval densities. Higher larval densities elevate temperature and aeration, stimulating metabolism and enzyme activity (**Chapter 1**) which may reduce the mortality of (young) larvae when starch is present in the excreta. In **Chapter 5** the hypothesis was tested that increasing larval density increases larval survival in starch containing excreta. Starch or glucose (control) were added to chicken excreta at 10% of total DM, followed by inoculation with ~8, 12, 16, or 20 housefly eggs per gram wet substrate. Larvae and substrate residues were harvested at 58 or 120 h to investigate effects on early larval survival and growth.

Most growth occurred by 58 h (70-90% of total increase in individual and total larval weight) and individual weight declined with density at both harvest times. Starch and glucose significantly increased individual weight at both harvest times, more strongly at low densities. At 58 h, total larval biomass increased with density, with glucose (+47%) and starch (+49%) showing significantly larger increases than starchless excreta (+15%). By 120 h, total larval biomass plateaued at low density in starchless excreta (+5%) but continued increasing with density for glucose (+64%) and starch (+53%). Total larval biomass in glucose and starch declined between harvests, coinciding with 50-60% substrate water loss. In starch-containing excreta, survival increased with density at both 58 h (+22%) and 120 h (+18%), which supports the **Chapter 5** hypothesis that higher larval densities enhance survival when starch is present.

However, increased survival and growth of the younger larvae (58 h harvest) with starch was somewhat surprising, knowing that it benefits older larvae the most (**Chapter 4**). Apparently young larvae were less negatively affected by starch when their density was high enough, potentially explained by positive effects of larger larval densities on ureolytic symbionts (uric acid emission,

substrate aeration) that locally increased ammonia and pH. As was the case with total biomass, 120 h survival might have been affected by the high water losses of the substrates. Dehydration might have penalized survival at 120 h with starch more than with glucose because starch is less bioavailable, potentially requiring more water for enzymatic breakdown. This idea is supported by general higher survival with glucose at 120 h. The **Chapter 5** outcomes suggest that an early harvest (58 h) of the highest larval density in 10% starch substrate gives the highest biomass yield, suggesting that early harvest of high densities could be a viable strategy in mass-rearing for bioconversion. But as mentioned, 120 h harvest showed a decline in survival and total biomass that is likely related to substrate moisture loss. Before 58 harvest of high larval densities can be recommended as a sensible strategy for larval mass-rearing, preventing dehydration of the substrate by adding water or increasing environmental humidity should be experimentally addressed.

To contextualize the present findings, comparisons can be made with the black soldier fly (BSF), a relatively well-studied species already applied in mass-rearing bioconversion systems (**Chapter 1**, Box 2). Several studies demonstrate clear density-dependent effects on BSF performance. Increasing density consistently reduces individual weight and prolongs development, while total biomass increases up to system-specific thresholds shaped by substrate and rearing conditions. For example, individual weight declined between 0.31 and 10 larvae cm² (Barragán-Fonseca *et al*. 2018, Dzepe *et al*. 2020), yet the larger number of larvae resulted in higher total biomass until a threshold of ~6.25 larvae cm², beyond which performance plateaued or declined (Yakti *et al*. 2022, Lopes *et al*. 2023). Optimal individual weights were observed at lower densities (~2 larvae cm^{-2}) depending on substrate quality (Nayak and Klüber 2025). Very high larval densities reduce individual weight and total biomass (Schreven *et al*. 2022, Klammsteiner *et al*. 2025). Overall, mid-range densities around ~5 larvae cm^{-2} tend to balance individual weight and total biomass most effectively (Cattaneo *et al*. 2024). The **Chapter 5** findings exhibited a clear trade-off between individual growth and collective yield, but in contrast to BSF, where biomass typically peaks at intermediate densities before declining, housefly biomass increased up to the highest tested density (20 eggs/g) without evidence of a clear optimum. Reductions in individual weight and survival at high density did not outweigh the effect of higher larval numbers, resulting in continued biomass gains. A likely explanation is that housefly larvae, with their much shorter development time and lower space requirements, experience weaker crowding constraints than BSF. And a trade-off larval density might be found by increasing density beyond 20 eggs/g.

6.5 Bioconversion efficiency across experiments

So far, the general discussion focused on evaluating housefly larval growth and survival. But the ultimate aim of this thesis is to explore improved DM and N bioconversion efficiency (BE_{DM}, BE_N) of animal manure by housefly larvae. Bioconversion efficiencies were calculated per container (pot or tray) as the absolute amount (g) of larval DM or N as a fraction (%) of the absolute amount (g) of DM or N present in the fresh larval substrate. Across all experiments, BE_{DM} and BE_N were, not surprisingly, strongly positively associated with total larval biomass (within-experiment correlations $r = 0.79\text{-}1.00$). The association was near-perfect in **Chapter 2**, **3** and **4** (all $r \geq 0.97$) and remained

strong in **Chapter 5** (BE_{DM} r = 0.81, BE_N r = 0.79). The BE_{DM} and BE_N outcomes of all chapter experiments are shown in Figure 2. Through its strong association with total biomass, both BE_{DM} and BE_N were positively associated with low doses (5-10%) of starch or glucose to (un)sterilized excreta (**Chapter 3**, **4** and **5**). The effects of the carbohydrates, particularly those of starch, were enhanced by later addition (72 h) during the larval growth period (**Chapter 4**) and early harvest (58 h vs. 120 h) of higher larval densities (**Chapter 5**). Heat sterilization of excreta mitigated negative effects of higher (>20%) starch concentrations and decreased both BE_{DM} and BE_N of pure excreta (**Chapter 2** and **3**).

Re-analysis of bioconversion data and treatment ranking

In the individual chapters, bioconversion outcomes were analyzed using generalized linear models (GLMs) with a beta distribution. The beta distribution is well suited for proportional responses bounded between 0 and 1 and when combined with a logit link, captures the skewness and heteroscedasticity of the bioconversion data. The chapter-specific GLMs included batch effects (inoculation date) as additional fixed effect to separate their variation from the residual error. Using batch effects as fixed and not as random effects is consistent with classical experimental design and avoids the identifiability and convergence issues associated with random-effect beta models.

While statistically optimal for within-chapter inference, the chapter-specific models have limitations for comparing bioconversion compared across chapters. First, in the original GLMs in **Chapter 3** (starch dose-response) and **Chapter 5** (larval density), starch dose and larval density were treated as categorical predictors with discrete levels. This choice avoided fitting smoothed curves on relatively small datasets characterized by irregular dips and plateaus. The disadvantage of this choice was that trends across levels could not be captured, limiting hard conclusions to tested doses and densities. Also, having multiple simple contrasts reduces statistical efficiency and hinders both within and between-chapter comparison of overarching response patterns. Second, in the individual analyses, relative change (%RC) was used both to compare treatments with their baseline and to compare differences between treatments. However, %RC is baseline-dependent and only suited for within-chapter interpretation as the same %RC can represent very different absolute changes. For example, a 50% increase could be an increase from 2 to 3% or from 10 to 15%.

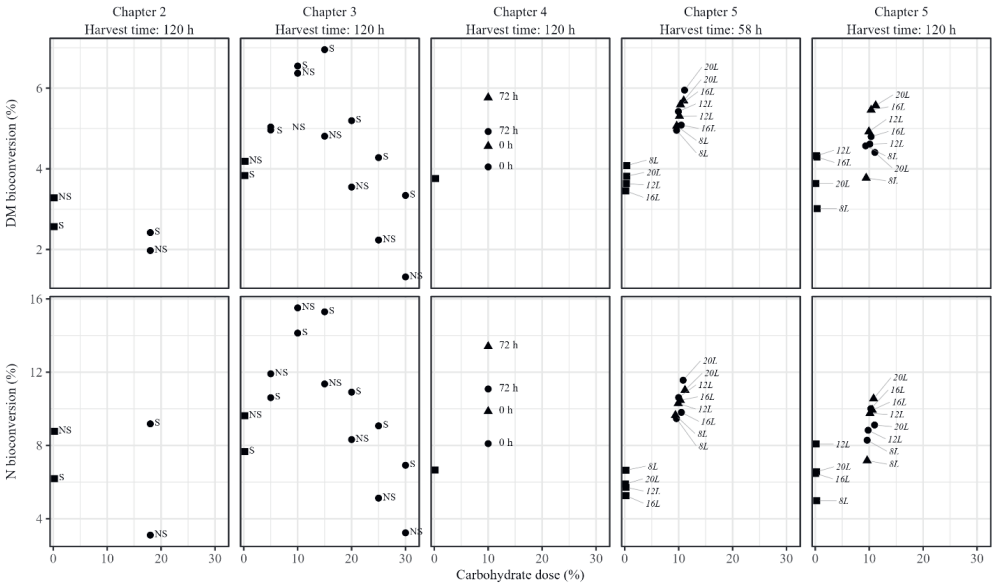


Figure 2. Dry matter (top row) and nitrogen (bottom row) bioconversion efficiency (%) per chapter experiment and carbohydrate dose (%). Columns show chapter experiments 2 to 5; harvest time is indicated below each column header. Shapes denote carbohydrate type: ■ = none, ● = starch, ▲ = glucose. Text labels indicate treatment facets: "S/NS" = e sterilized / non-sterilized excreta (Chapter 2-3); "0 h/72 h" = carbohydrate addition time (Chapter 4); "8L-20L" = larval density (Chapter 5). Points are means of replicate units, minor horizontal jitter was used to improve readability. Starch treatments > 30% were omitted by design.

To enable a more systematic comparison of bioconversion outcomes across chapters, the raw data from each chapter were compiled and reanalyzed using a single framework based on generalized additive models (GAMs) with Gaussian error distributions. The GAMs allow for different experimental designs (simple contrasts, time pairs, continuous slopes and curves) while using a consistent contrast-estimation procedure, resulting in effect estimates on a common scale.

In the re-analysis, starch dose (**Chapter 3**) and larval density (**Chapter 5**) were treated as continuous predictors, allowing smoother trends to be fitted across their full ranges and facilitating identification of general response patterns beyond the discrete levels used in the original analyses. For **Chapter 3**, the data were restricted to starch concentrations up to 20%, as higher doses consistently showed strong negative effects. To reduce the influence of local dips and plateaus on fitted trends for starch dose and larval density, GAMs were fitted using restricted maximum likelihood. Batch variation (inoculation day) was not included in the GAMs because it was small and statistically non-significant in all chapter models and thus unlikely to affect cross-chapter inference. Effect estimates derived from the GAMs are expressed in percentage points (pp), representing absolute differences in bioconversion that can be directly compared across chapters (Figure 3). These estimates are intended to show consistent patterns and relative effect magnitudes across chapters, rather than to replace experiment-specific inference presented in the individual chapters.

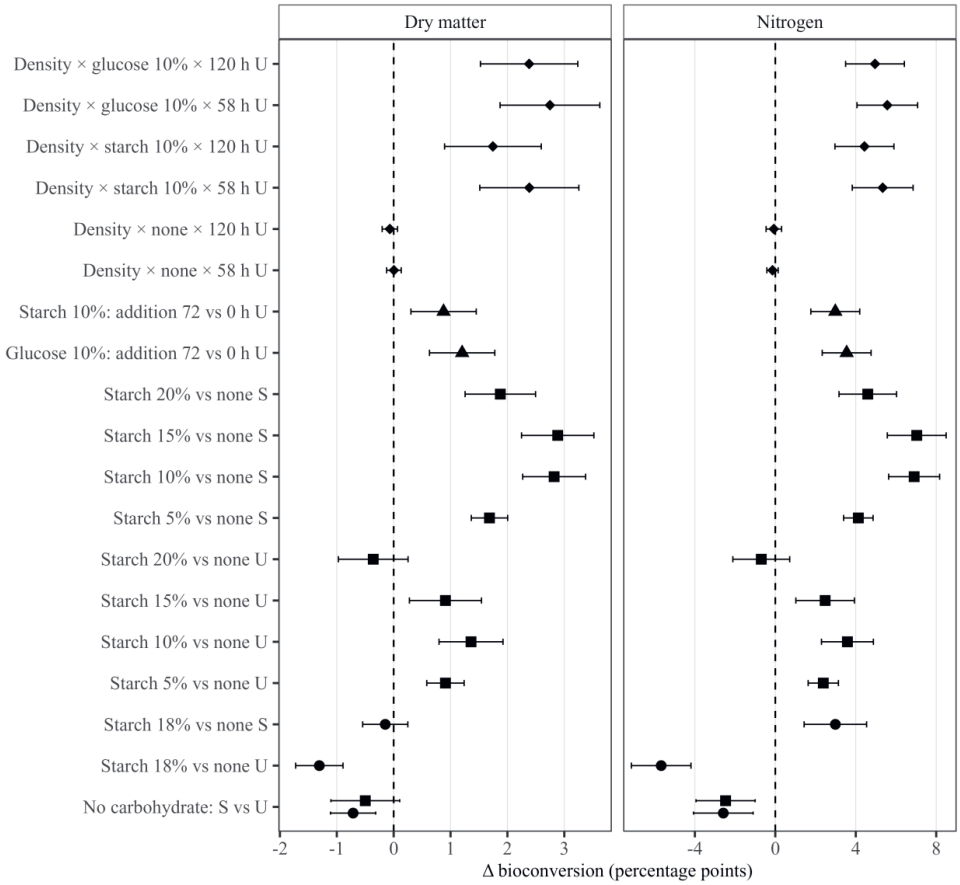


Figure 3. Estimated treatment effects (Δ bioconversion percentage points ±95% CI) for substrate dry matter and nitrogen bioconversion efficiency across the four thesis chapters. Effects are expressed as contrasts relative to baseline conditions: no carbohydrate at the same sterilization state, 120 h harvest and 8 larvae/g substrate. Chapter 5 density contrasts show the effect of a +5 larvae/g substrate increase within each carbohydrate × harvest condition. U = unsterilized; S = sterilized. Shapes denote chapter experiments: ● Chapter 2, ■ Chapter 3, ▲ Chapter 4, ◆ Chapter 5. Model fit: experiment 1 n = 23; R^2(DM/N) = 0.63/0.78; experiment 2 n = 66; R^2(DM/N) = 0.95/0.95; experiment 3 n = 24; R^2(DM/N) = 0.59/0.76; experiment 4 n = 121; R^2(DM/N) = 0.76/0.88.

Without starch and at an equal larval density (8 larvae/g), sterilization of excreta lowered BE_{DM} and BE_N relative to unsterilized excreta. The re-analysis showed reductions of -1.3 pp (DM) and -2.6 pp (N) for **Chapter 2** and -0.5 pp (DM) and -2.5 pp (N) for **Chapter 3**.

Using the criterion that a treatment mean exceeding the 95% confidence interval of another indicates a significant difference ($p < 0.05$), treatments can now be ranked. The strongest increases in BE_{DM} are seen for increased larval density in unsterilized excreta with 10% glucose (+3.31 pp [2.07-4.55]) or 10% starch (+3.29 pp [2.04-4.54]), followed closely by sterilized excreta containing 15% starch at

8 larvae/g and 120 h harvest (+3.12 pp [2.23-4.01]). The optimal **Chapter 3** 10% starch concentration in unsterilized excreta at 8 larvae/g (+2.18 pp [1.29-3.07]) ranks below this top three but outperforms the 5% (+0.85 pp [-0.04 to -1.74]), 15% (+0.62 pp [-0.27-1.51]) and 18% starch (-1.31 pp [-1.64 to -0.97]) in unsterilized excreta with 8 larvae/g and 120 harvest. The best treatments to increase BE_N were using sterilized excreta with 15% starch (+7.62 pp [5.86-9.39]) and 10% starch (+6.46 pp [4.69-8.23]) at 8 larvae/g and harvested at 120 h. These were closely followed by increased larval density in unsterilized excreta with 10% starch, harvested at 58 h (+6.12 pp [3.84-8.4]). As for BE_{DM}, the optimal **Chapter 3** 10% starch in unsterilized excreta with 8 larvae/g and 120 harvest (+5.89 pp [4.12-7.66]) was lower but close to the top treatments.

The optimal treatments differ for BE_{DM} and BE_N, with excreta sterilization being more important for the latter. This might reflect involvement of different biological and chemical processes. It appears that BE_{DM} is primarily driven by energy availability and larval growth rate, which are maximized under high larval densities and early harvest in unsterilized excreta. In contrast, BE_N is more strongly influenced by microbial competition and possibly nitrogen loss pathways, with the highest nitrogen retention occurring under sterilized conditions with high starch supplementation.

A similar decoupling, with treatments yielding the highest BE_{DM} differing from those maximizing BE_N, is documented in black soldier fly (*Hermetia illucens*) culture, where carbohydrate-rich diets strongly increase larval dry mass but, by disproportionately promoting lipid accumulation, result in lower protein yield relative to total biomass gain (Barragán-Fonseca *et al*. 2017, Spranghers *et al*. 2017). Analogous trade-offs also occur in microbial bioprocessing, where conditions optimized for rapid biomass production often reduce nitrogen use efficiency due to overflow metabolism and by-product formation (Russell and Cook 1995). Likewise, in composting and manure treatment systems, strategies that accelerate organic matter degradation (addition of easily digestible carbohydrates) typically increase substrate nitrogen losses unless microbial activity and substrate composition are carefully controlled (Bernal *et al*. 2009).

6.6 Limitations and methodological considerations

Repeatability and reproducibility

The cross-experiment dataset used to recalculate and rank bioconversion was also used to assess repeatability and reproducibility. Definitions of repeatability and reproducibility follow ISO 5725 (1994), where repeatability refers to precision under identical conditions and reproducibility refers to precision under varying conditions such as different experimental runs. Repeatability was quantified using coefficients of variation (CV), calculated across replicate containers within experiment × treatment combinations as the standard deviation divided by the mean and expressed as a percentage. Coefficients of variation were calculated for treatment combinations with at least two replicate containers. Distributions of CVs were summarized using median, interquartile range (IQR) and full range to show typical repeatability and (biologically induced) extremes. Though the primary focus was bioconversion efficiency, repeatability of individual larval mass was included to aid

interpretation of bioconversion estimates, which are ratios and affected by amplification of measurement variability from both numerator and denominator.

Reproducibility across experimental runs was assessed using intraclass correlation coefficients (ICC). On a scale between 0 and 1, the ICC quantifies how much of the total variability in the data is due to differences between experimental runs, as opposed to differences among containers within the same run. For bioconversion efficiency and individual larval mass measured under identical treatments occurring in two or more experiments, ICCs were derived from one-way random-effects models with experimental run specified as a random intercept and no fixed effects. Four identical treatment combinations occurred in two or more experiments: (i) unsterilized starchless excreta with 8 larvae / g wet substrate harvested at 120 h (**Chapter 2**,**3**,**4** and **5**); (ii) sterilized starchless excreta with 8 larvae/g wet substrate harvested at 120 h (**Chapter 2** and **3**); (iii) unsterilized excreta with 10% starch added at 0 h, 8 larvae/g wet substrate and harvested at 120 h (**Chapter 2** and **4**) and (iv) unsterilized excreta with 10% glucose added at 0 h, 8 larvae/g wet substrate and harvested at 120 h (**Chapter 4** and **5**).

Median CVs were below 10% for all response variables (Table 1), a range commonly considered indicative of good precision in analytical chemistry. Median CVs for BE_{DM}, BE_N and individual larval mass were comparable all below 10% and with overlapping interquartile ranges (Table 1). These results show that BE is not inherently more variable at the level of replicate containers than individual larval mass, suggesting that effect of error multiplication mentioned above is small/limited. The full ranges of CVs did reveal extreme values for BE that were much less present in individual larval mass. These were primarily found for treatment combinations yielding very low mean bioconversion, notably at high (> 30%) starch supplementation levels in **Chapter 2**, where larval biomass was minimal and small absolute differences among replicate containers translated into large relative variation.

The ICC values showed a large contribution of between-experiment variation to total variability for individual larval mass and BE, with values being closer to one than to zero (Table 1). Reproducibility differed between supplemented and starchless excreta and within supplemented excreta. For starchless excreta, ICC values were high for both individual larval mass (0.95) and BE_N (0.96), whereas BE_{DM} showed a lower ICC (0.64). For 10% starch, ICC values were 0.68 for individual larval mass, 0.91 for dry-matter bioconversion efficiency and 0.97 for nitrogen bioconversion efficiency. For 10% glucose, corresponding ICC values were 0.60, 0.73, and 0.87, respectively. For both carbohydrates, individual larval mass exhibited a smaller between-experiment component than both BE, while BE_N consistently showed the strongest between-experiment variation. The high ICC values are not unexpected, as they primarily reflect differences between experimental runs. Different chapters address different research questions and use different excreta batches and experimental scales (100-500 g). Experiment preparation (excreta manipulation, housefly egg production, substrate inoculation with eggs) were largely consistent as the within-run variability was moderate to low.

Table 1. Repeatability (CV, %) and reproducibility (ICC) of nitrogen (BE_N) and dry matter (BE_{DM}) bioconversion efficiency and individual larval mass across experiments. ICCs are reported only for treatments shared by two or more experiments; replication is shown as number of experiments / number of containers (N / n).

Assessment	Response variable	CV median (%)	CV IQR (%)	CV range (%)	ICC	Replication (N/n)
Repeatability	BE_{DM}	9.5	6.1-17.6	2.7-94.5	-	-/54[1]
	BE_N	8.4	5.4-18.2	0.8-92.8	-	-/54[1]
	Individual larval mass	8.8	4.8-15.2	1.7-55.2	-	-/54[1]
Reproducibility	BE_{DM} (none)	-	-	-	0.64	4/24[2]
	BE_N (none)	-	-	-	0.96	4/24[2]
	Individual larval mass (none)	-	-	-	0.95	4/24[2]
	BE_{DM} (starch 10%)	-	-	-	0.91	3/18[2]
	BE_N (starch 10%)	-	-	-	0.97	3/18[2]
	Individual larval mass (starch 10%)	-	-	-	0.68	3/18[2]
	BE_{DM} (glucose 10%)	-	-	-	0.73	2/12[2]
	BE_N (glucose 10%)	-	-	-	0.87	2/12[2]
	Individual larval mass (glucose 10%)	-	-	-	0.60	2/12[2]

[1] N = absent, no experiment-level replication, n = number of individual CV estimates based on ≥2 containers.

[2] N = number of experiments (chapters), n = number of individual containers.

Typically, (published) insect growth and bioconversion studies do not report CVs or ICCs. Repeatability and reproducibility are usually assessed implicitly through visual comparison of replicate means or trends, rather than by explicit partitioning of variability. This contrasts with the fact that these metrics are standard practice in industry (animal production, feed production, fermentation). Academic research is explorative and prioritizes treatment effects within single experimental runs by means of hypothesis testing using the lowest sample size calculated, or deemed, adequate. Unless of specific interest, batch variation is a nuisance, whereas industry prioritizes the management of variability at the run level, for which unit-free measures like CV and ICC are particularly informative. Nevertheless, it would be good practice to incorporate reproducibility in experimental design of insect bioconversion studies as these might increasingly be used to inform scale-up and application.

In housefly manure bioconversion studies, the use of a single excreta batch would substantially improve reproducibility. Standardizing the substrate would not only equalize starting conditions such as nutrient content, moisture, age and microbiota, but would also allow better diagnosis of remaining between-run differences (temperature, handling day, egg hatch variability, scale). An alternative strategy to improve reproducibility would be intentional repetition of identical treatments across experimental runs. However, this would be at a trade-off with the number of samples needed to study treatment effects. In the thesis experiments, practical constraints limited throughput to 24-30 containers per day by a single operator. This limit was mainly set by the combined demands of substrate preparation and egg manipulation. Both egg harvesting and egg partitioning must be completed within a narrow time window (3-5 h) to prevent egg ageing and desiccation, not allowing

for parallel tasks. Consequently, increasing replication beyond this level would require either additional operators or a fundamental change in workflow. The latter could involve incorporating substrate and replicate preparation into the initial fresh excreta handling. Rather than homogenizing and freezing bulk excreta that must later be thawed, supplemented and portioned at the start of each experiment, replicate containers could be prepared immediately after homogenization and frozen as ready to use experimental units.

Excreta homogenization

The experiment described in **Chapter 2** pointed to the need for homogenizing chicken excreta which was used for all later experiments. Homogenization greatly improved excreta manageability as experimental material. Fresh excreta consists of discrete droppings that tend to clump together during transport and storage, forming a clay-like mass. This consistency makes it very difficult to create uniform mixtures with powdered supplements. This was not evident in preliminary tests using small amounts of excreta (<100 g) spread in thin layers. Homogenization with a meat cutter or equivalent apparatus that both cuts and mixes *without compressing* the material leads to a fluffy homogenous material that is much easier stirred. This allows for a much more uniform distribution of powdered additives in the excreta. With some experimentation, mixing in powdered additives could be part of the homogenization process.

Estimation of larval survival

In **Chapter 3**, the relation between starch concentration and mean individual or total biomass followed an asymmetric curve, peaking before declining, whereas survival appeared highly linear. This discrepancy partly reflects the statistical properties of larval weight distributions. Survival was estimated as total biomass divided by mean individual weight, which is unbiased only if weights are normally distributed. But unfavourable conditions, as found in unsterilized excreta with high starch concentrations (**Chapter 2** and **3**) or when high densities of larvae are present (**Chapter 5**) tend to generate skewed distributions of individual weight. Here the proportion of lighter larvae is larger or smaller than that of heavier larvae, often also showing kurtosis (rare very heavy larvae). The distributions seen in **Chapter 3** supplementary material are the best example of this. For 0-15% starch, distributions are highly normal (mean ~ median, skewness ~ 0). In this case, survival is an unbiased estimate as it is not affected by skew of the denominator (mean individual weight). At 15-20% starch in sterilized excreta, left-skew occurs (mean > median, skewness < 0) as a minority of heavier larvae pulls the mean above the median. So in the survival calculation, the denominator has increased relative to the most common larva weight and survival is underestimated as dividing biomass by a larger mean gives fewer implied survivors. At > 25% starch, predominantly in unsterilized excreta, distributions become right-skewed, many small larvae pull the mean below the median. The denominator decreases relative to the most common larva weight and dividing biomass by a smaller mean inflates survival. So here survival is overestimated, with numerous tiny larvae contributing little to the larval biomass. Thus, while biomass accurately reflects the combined effects of survival and individual weight, the survival metric is mathematically biased. The opposing biases

at moderate and high starch concentrations (left- vs right-skew) flatten the curve, making survival appear more linear than it truly is.

In theory, the gamma models used to calculate mean individual weights should have reduced skew-related bias as they estimate the mean as the central tendency of a fitted distribution (over the low, mean and high larvae count), rather than a raw average, thereby down-weighting the influence of rare very heavy or very small larvae. However, the resulting **Chapter 3** survival curves are still linear. This could be caused by several reasons that are not resolved by using a skew-moderating distribution for the denominator. It could be that, because biomass and mean weight share similar curvilinear responses to starch and when survival is calculated as their ratio, the common curvature cancels out and the trend appears linear. Also, the gamma fit stabilizes the denominator by smoothing extremes, damping nonlinearity. And survival itself may respond more gradually to starch than individual weight and total biomass, so even after statistical correction the underlying biological trend remains close to linear. A straightforward solution would be to use precisely known egg numbers, so that survival can be calculated directly as the proportion developing into larvae. However, obtaining exact counts is quite difficult because the eggs are tiny (~0.07 mg) and tend to clump (**Appendix 2**). A promising approach may be to disperse them in a single water layer and count them by image analysis.

Starch source

All experiments in this thesis used gelatinized corn starch, which is highly digestible to both larvae and microorganisms. This is useful for initial experimentation and controlled comparisons but limits generalization to real world starch (waste) streams, in which starch is raw, partially degraded or mixed with proteins, fibres or fats. Thus, the potential of starch-containing by-products as excreta supplement will differ depending on botanical origin, structure and processing. For example, cereal by-products such as brewery spent grains and bakery waste contain both starch and protein, while root and tuber sources like cassava and potato vary in amylose-to-amylopectin ratios and legume-based starches often carry resistant starch fractions (Artavia-Granados *et al*. 2020, Feng *et al*. 2024, Rashwan *et al*. 2024,Šárka *et al*. 2025). Apart from having a different digestibility and impact on (microbiota of) the housefly-manure system, these available starch-waste streams can also be used for poultry and pig feed. To avoid this competition and use housefly larvae bioconversion as part of true circular agriculture, suitable starch sources need to be identified.

6.7 Implications for chicken excreta nutrient upcycling

To place the experimental findings in an applied context, a simplified hypothetical scenario can be used to illustrate the potential implications for excreta N upcycling. Assuming a DM content of 50% and an average 4% N content (of DM), one metric ton of fresh chicken excreta contains 500 kg of dry matter and 20 kg of N. Using the simplest representative experimental parameters, unsterilized starchless excreta, a larval density of 8 larvae/g and a harvest time of 120 h, the across-experiment average BE_N of approximately 8% would result in about 1.6 kg of excreta N being incorporated into larval biomass. This corresponds to an estimated larval protein yield of roughly 10 kg per ton of fresh excreta (~20 kg larval dry matter, assuming 50% crude protein in larvae and crude protein as 6.25×N). In contrast, when excreta are supplemented with 10% starch (of DM) using the same experimental parameters, BE_N could increase to approximately 12%, which would yield about 2.4 kg of larval N and an estimated 15 kg of protein per ton of fresh excreta (~30 kg larval dry matter).

The total national livestock manure production in the Netherlands in 2021 was approximately 73.4 million tonnes, of which around 5-6% was attributable to poultry and related birds (CBS 2023, CLO 2023). This proportion implies an amount of roughly 4-5 million tonnes of poultry manure in 2021. Assuming all poultry manure consists of fresh excreta and scaling the found BE_N under the assumptions used above to this national poultry manure volume, the conversion factors imply tens to hundreds of thousands of tonnes of insect protein potential. For example, at a conservative estimate of 4 million tonnes of fresh poultry excreta per year, a 6% BE_N rate could yield on the order of 30,000 tonnes of crude larval protein, whereas a 12% BE_N rate associated with starch supplementation could approach 60,000 tonnes of crude insect protein annually.

6.8 Suggestions for future research

Supplementation with relatively low doses of starch was found effective to increase larval growth on and bioconversion of fresh chicken excreta. But this thesis did not address amino acid profiles or digestibility of the protein present in chicken excreta, nor did it clarify what part of excreta larvae actually ingest, whether the relevant protein source consists of dead bacterial material or of live bacteria that grow post-defecation and if the type of material ingested indeed has a temporal component. It is unknown what fraction of manure actually contains the protein and other nutrients such as essential vitamins and minerals. Nutrient analysis of excreta fractions, resulting from water extraction combined with sieving, filtering or particle sedimentation can potentially answer this question.

Knowing in what excreta fraction, in which form (free or incorporated in bacterial cells) nutrients occur could also address the problem of variability in the raw excreta, yielding criteria to determine suitability of different batches and/or collection processes. This knowledge should lead to conservation/storage methods for excreta as excreta ages quickly at ambient temperatures in aerobic conditions. Unless housefly mass-rearing systems are integrated closely with poultry production, transport and storage of excreta will lead to deterioration of its nutritional value. Possible conservation strategies could be drying and re-hydrating excreta before use, anaerobic storage preventing microbial

succession or, mimicking the larvae-microbiota interaction, strongly increasing the pH by adding bases, such as calcium hydroxide, that are tolerated by larvae. Using the symbiotic bacteria (**Chapter 1**, Box 1) as a probiotic conservation strategy also has potential.

Identifying the optimal microbiota should be a paramount element of future optimization research. Experiments that combine high-throughput sequencing and different larval densities will be able identify the optimal microbiota that form part of the larvae-manure interaction. This will also generate insight on the effect of larvae themselves on the microbiota.

Developing the analysis of volatiles (odor) generated by the manure-larvae system might be a valuable approach for both research purposes and industrial in-process control. My subjective experience with hundreds of samples during larvae rearing and manure experiments was that good and bad substrates, both dog food and excreta based, have a characteristic odor. This odor is prone to be a more or less complex mixture of different volatile compounds but it should be possible to identify key compounds with gas chromatography and/or mass spectrometry. Associating these key compounds with the optimal bioconversion outcomes of the larvae-manure system or parameters such as substrate heat production could allow for process monitoring without the need to take and analyze substrate samples in other ways (pH, ammonia, microbiota sequencing).

In order to supplement manures with starch, suitable starch waste streams should be identified. These should fit into the nutritional ecology of larvae in excreta, so as to not negatively affect functional microbiota and not compete with other animal feed uses. When identifying such a waste stream, a crude criterion could be the ability to maintain or prolong the alkaline-ureolytic window in excreta that appeared optimal for (early) larval survival and growth.

The use of larval stage-dependent substrates deserves systematic testing. The experimental results indicate that early larval survival might be key to subsequent harvestable larval biomass. Developing a rearing system in which the youngest larvae use a high protein starchless substrate, such as brewer's yeast or fish waste, possibly inoculated with larval symbionts, followed by transfer to starch containing excreta, may reduce early-stage mortality and enhance overall growth performance by matching diet composition to age-dependent nutritional needs.

Engineering aspects of mass-rearing will also need substantial investigation. Transferring laboratory methods that work at a scale of less than a kilo to production facilities that utilize hundreds of tonnes of excreta needs optimization of especially substrate moisture and heat management. Also, down-process methods for large scale harvest including cleaning of larvae do not yet exist, at least not in published literature. The salt water harvesting method developed as part of this thesis (**Appendix 1**) has potential for scale-up and automatization.

6.9 Main conclusions

1) Starch supplementation of fresh chicken excreta increases larval biomass and DM and N bioconversion only within a narrow concentration window determined by microbial thresholds. Beyond this concentration window, small increases in starch concentration trigger sharp declines in larval survival and biomass and abrupt shifts in substrate chemistry driven by microbial activity. These findings demonstrate that larval growth on excreta is constrained by microbially mediated threshold effects, rather than responding linearly to starch supplementation.

2) Fresh chicken excreta is energy-limited for larval growth, but starch has both beneficial and detrimental effects. In the absence of digestible carbohydrates, larval growth on fresh chicken excreta is limited by insufficient available energy, restricting efficient utilization of dietary protein. Low doses of starch or glucose (~5-10% DM) mitigate this limitation and consistently increase larval biomass and bioconversion efficiency. However, more than glucose, starch appears to stimulates microorganisms, leading to reduced larval survival through intensified microbial competition and/or modification of essential larval growth conditions.

3) Larval survival and early growth depend on alkaline substrate conditions. High larval biomass coincides with alkaline conditions and high ammonia concentrations, likely driven by ureolytic bacteria. Excess starch pushes the system toward acidogenic conditions characterized by a lower pH and decreased larval survival. As stated under point 1, this microbial transition represents a threshold phenomenon, not a gradual response.

4) Heat sterilization shifts, but does not remove, microbial effects on larval growth outcomes. Sterilization reduces initial microbial competition and raises tolerance to higher starch concentrations, shifting the starch threshold upward.

5) Timing of carbohydrate addition is important, starch is best supplied later during the larval growth period. Early starch addition reduces larval survival, whereas late starch supplementation (at 72 h) increases larval survival and biomass. This shows that larval nutritional or substrate requirements change over development, with young larvae being particularly sensitive to starch-driven microbial shifts, while older larvae can use starch more safely.

6) High larval densities mitigate starch-induced mortality. Increasing larval density substantially improves survival in starch-supplemented excreta. Likely mechanisms include enhanced local alkalinization, aeration, heat production and symbiont activity.

7) Bioconversion efficiency closely tracks total larval biomass, but DM and N recovery diverge. Dry matter and nitrogen bioconversion efficiencies are strongly correlated with larval biomass, yet optimal conditions differ. When supplementing excreta with 10% starch, dry matter bioconversion is maximized by high density, early harvest and unsterilized substrates while nitrogen bioconversion is maximized by excreta sterilization.

References

Artavia, G., Cortés-Herrera, C., Granados-Chinchilla, F. (2020). Total and resistant starch from foodstuff for animal and human consumption in Costa Rica. *Current Research in Food Science*, 3, 275-283.

Barragán-Fonseca, K. B., Dicke, M., van Loon, J. J. A. (2017). Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed: A review. *Journal of Insects as Food and Feed*, 3(2), 105-120.

Barragán-Fonseca, K. B., Dicke, M., van Loon, J. J. A. (2018). Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (*Hermetia illucens*). *Entomologia Experimentalis et Applicata*, 166(9), 761-770.

Bernal, M. P., Alburquerque, J. A., Moral, R. (2009). Composting of animal manures and chemical criteria for compost maturity assessment: A review. *Bioresource Technology*, 100(22), 5444-5453.

Blahovec, J., Kostecka Z., Kocisova A. (2006). Peptidolytic enzymes in different larval stadium of house fly *Musca domestica*. *Veterinarni Medicina*, 51, 139-144.

CBS 2023. Livestock manure; production, transport and use, key figures [Data set].

Cattaneo, C., van Huis, A., Dicke, M. (2024). The impact of larval density on growth, development, and bioconversion efficiency in black soldier fly larvae (*Hermetia illucens*): A review. *Insects*, 15(2), 152.

Chaucheyras-Durand, F., Durand, H. (2010). Probiotics in animal nutrition and health. *Beneficial Microbes*, 1(1), 39.

CLO 2023. Mestproductie door de veestapel. https://www.clo.nl/indicatoren/nl010431-mestproductie-door-de-veestapel

Cutting, S. M. (2011). Bacillus probiotics. *Food Microbiology*, 28(2), 214-220.

de Jonge, N., Michaelsen, T. Y., Ejbye-Ernst, R., Jensen, A., Nielsen, M. E., Aamand, J. (2020). Housefly (*Musca domestica* L.) associated microbiota across different life stages. *Scientific Reports* 10, 7842.

Dou, Y., Yu, X., Luo, Y., Chen, B., Ma, D., Zhu, J. (2022). Effect of fructooligosaccharides supplementation on the gut microbiota in human: A systematic review and meta-Analysis. *Nutrients*, 14(16), 3283.

Dzepe, D., Nana, P., Kekeunou, S., Tchuenguem, F.-N., Messi, J. (2020).. Effect of larval density on the growth and development of the black soldier fly (*Hermetia illucens*) (Diptera: Stratiomyidae). *Journal of Insects as Food and Feed*, 6(3), 237-245.

Feng, H., Cheng, B., Lim, J., Li, B., Li, C., Zhang, X. (2024). Advancements in enhancing resistant starch type 3 (RS3) content in starchy food and its impact on gut microbiota: A review. *Comprehensive Reviews in Food Science and Food Safety*, 23(3), e13355.

Freese, E., Sheu, C. W., Galliers, E. (1973). Function of lipophilic acids as antimicrobial food additives. *Nature*, 241(5388), 321-325.

Hadinoto, K., Ling, J. K. U., Pu, S., Tran, T. T. (2024). Effects of alkaline extraction pH on amino acid compositions, Protein Secondary Structures, Thermal Stability, and Functionalities of Brewer's Spent Grain Proteins. *International Journal of Molecular Sciences*, 25(12), 6421.

ISO 5725. International Organization for Standardization. (1994). Accuracy (trueness and precision) of measurement methods and results. Part 1: General principles and definitions (ISO 5725-1). Geneva, Switzerland.

Klammsteiner, T., Apprich, S., Insam, H., Ertl, P. (2025). Density-dependent thermogenesis and microbiota shifts in black soldier fly larvae (*Hermetia illucens*). *Science*, 24(6), 102587.

Kwan, Y. H., Tung, Y. K., Kochhar, J. S., Li, H., Poh, A.-L., Kang, L. (2014). Essential monographs. In Y. H. Kwan, Y. K. Tung, J. S. Kochhar, H. Li, A.-L. Poh, L. Kang (Eds.), Handbook of cosmeceutical excipients and their safeties (pp. 75-314). Cambridge, UK: Woodhead Publishing.

Lin, S. W., Shelomi, M. (2024). Black soldier fly (*Hermetia illucens*) microbiome and microbe interactions: a scoping review. *Animals*, 14(22), 3221.

Lopes, I. G., Lalander, C., Vidotti, R. M., Vinnerås, B. (2023). Larval density and feed depth affect black soldier fly (*Hermetia illucens*) larval growth and feed conversion in small-scale systems. *Waste Management*, 152, 87-95.

Metcalfe, N. B., Valdimarsson, S. K., Morgan, I. J. (2003). The relative roles of domestication, rearing environment, and prior experience in the development of hatchery performance in Atlantic salmon (*Salmo salar*). *Journal of Fish Biology*, 62(3), 517-533.

Metzler-Zebeli, B. U., Lawlor, P. G., Magowan, E., McCormack, U. M., Curião, T., Hollmann, M., Ertl, R., Aschenbach, J. R., Zebeli, Q. (2017). Finishing pigs that are divergent in feed efficiency show small differences in intestinal functionality and structure. *PLOS ONE,* 12(4), e0174917.

Mobley, H. L., Island, M. D., Hausinger, R. P. (1995). Molecular biology of microbial ureases. *Microbiological Reviews*, 59(3), 451-480.

Morath, S. U., Hung, R., Bennett, J. W. (2012). Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biology Reviews*, 26(2-3), 73-83.

Morita, T., Kasaoka, S., Ohhashi, A., Ikai, M., Numasaki, Y., Kiriyama, S. (1998). Resistant proteins alter cecal short-chain fatty acid profiles in rats fed high amylose cornstarch. *Journal of Nutrition*, 128(7), 1156-1164.

Nayak, S. K., Klüber, P. (2025). Effects of larval density and substrate moisture on growth and waste reduction by black soldier fly larvae (*Hermetia illucens*). *PLOS One*, 20(2), e0300123.

Pei, Y., Zhao, S., Chen, X., Zhang, J., Ni, H., Sun, M., Lin, H., Liu, X., Chen, H., Yang, S. (2022). *Bacillus velezensis* EEAM 10B strengthens nutrient metabolic process in black soldier fly larvae (*Hermetia illucens*) via changing gut microbiome and metabolic pathways. *Frontiers in Nutrition*, 9, 880488.

Poulaki, E. G., Tjamos, S. E. (2023). Bacillus species: factories of plant protective volatile organic compounds. *Journal of Applied Microbiology*, 134(3), lxac138.

Rashwan, A. K., Younis, H. A., Abdelshafy, A. M., Osman, A. I., Eletmany, M. R., Hafouda, M. A., Chen, W. (2024). Plant starch extraction, modification, and green applications: a review. *Environmental Chemistry Letters*, 22(5), 2483-2530.

Russell, J. B., Cook, G. M. (1995). Energetics of bacterial growth: Balance of anabolic and catabolic reactions. *Microbiological Reviews*, 59(1), 48-62.

Sakr, S. F., Gabr, H. R. (2018). Length-weight relationship and condition factor of Nile tilapia (*Oreochromis niloticus*) from the Nile River, Egypt. *Egyptian Journal of Aquatic Research*, 44(4), 303-308.

Šárka, E., Dvořáček, V., Horák, V. (2025). Crystallinity of starch, food composition, and digestibility of starch. *Czech Journal of Food Sciences*, 43, 1-10.

Schreven, S. J. J., de Smet, J., Bosch, G., Dicke, M., van Loon, J. J. A. (2022). Impact of larval density on performance, microbial community, and resource use of black soldier fly larvae (*Hermetia illucens*) reared on different substrates. *Applied and Environmental Microbiology*, 88(9), e02477-21.

Shivaramaiah, S., Pumford, N. R., Morgan, M. J., Wolfenden, R. E., Wolfenden, A. D., Torres-Rodríguez, A., Hargis, B. M., Téllez, G. (2011). Evaluation of *Bacillus* species as potential candidates for direct-fed microbials in commercial poultry. *Poultry Science*, 90(7), 1574-1580.

Smid, E. J., Kleerebezem, M. (2014). Production of aroma compounds in lactic fermentations. *Annual Review of Food Science and Technology*, 5, 313-326.

Stefanelli, C., Pinna, C., Biagi, G., Zaghini, G., Nannoni, E., Grandi, M., Vecchiato, C. (2016). *In vitro* influence of dietary protein and fructooligosaccharides on metabolism of canine fecal microbiota. *BMC Veterinary Research*, 12(1), 1-9.

Strachan, C. R., Law, R. J., Rattray, F. P., McSweeney, P. L. H., McAuliffe, O. (2025). Distinct lactate utilization strategies drive niche differentiation. *The ISME Journal*, 19(1), 1-13.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Advances in Immunology*, 121, 91-119.

Thorpe, J. E., Mangel, M., Metcalfe, N. B., Huntingford, F. A. (1998). Modelling the proximate basis of salmonid life-history variation, with application to Atlantic salmon, *Salmo salar* L. *Evolutionary Ecology*, 12, 581-599.

Tiquia, S. M., Tam, N. F. Y. (2000). Fate of nitrogen during composting of chicken litter. *Environmental Pollution*, 110(3), 535-541.

Topping, D. L., Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews*, 81(3), 1031-1064.

Wang, Y.-L., Wang, W.-K., Wu, Q.-C., Zhang, F., Li, W.-J., Yang, Z.-M., Yang, H.-J. (2022). The effect of different lactic acid bacteria inoculants on silage quality, phenolic acid profiles, bacterial community and *in vitro* rumen fermentation characteristic of whole corn silage. *Fermentation*, 8(6), 285.

Yakti, R., Bosch, G., van Loon, J. J. A., van Huis, A. (2022). Influence of larval density on growth and performance of black soldier fly larvae (*Hermetia illucens*) at two production scales. *Sustainability*, 14(13), 8011.

Zhang, Y., Liu, Y., Li, J., Xing, T., Jiang, Y., Zhang, L., Gao, F. (2020). Dietary resistant starch modifies the composition and function of caecal microbiota of broilers. *Journal of the Science of Food and Agriculture*, 100(3), 1274-1284.

Zhao, Y., Wang, W., Zhu, F., Wang, X., Wang, X., Lei, C. (2017). The gut microbiota in larvae of the housefly *Musca domestica* and their horizontal transfer through feeding. *Applied Microbiology Express*, 7, 147.

Zurek, L., Schal, C., Watson, D. W. (2000). Diversity and contribution of the intestinal bacterial community to the development of Musca domestica larvae. *Journal of Medical Entomology*, 37(6), 924-928.

Summary

Summary

Housefly larvae have a high potential for the bioconversion of fresh animal manures, upcycling nutrients and contributing to (surplus) manure management. Fresh animal manures have a relatively high protein content but contain little to no digestible carbohydrates. Although housefly larvae can fully develop on pure fresh manure, their facultative ability to digest starch suggests that starch supplementation is a viable option to increase larval yield and manure bioconversion. In pure manures larvae use protein as an energy source and shifting the protein-energy balance of their diet by addition of energy (starch) might decrease larval competition for protein, leading to higher survival of bigger larvae, increase more efficient use of protein (higher protein deposition) and decreasing nitrogenous waste from larval protein catabolism that would be lost by degradation to and volatilization of ammonia. However, fresh manure has a high microbial load and addition of easily digestible carbohydrates such as starch could lead to changes in microbial diversity and abundance that negatively affect larval survival and growth. In **Chapter 1**, a synthesis was made of available information, combining information on larval nutritional needs, digestive physiology, housefly phylogeny and larval ecology to identify knowledge gaps and testable hypotheses/strategies for suppletion of fresh manure with starch. In **Chapter 2**, the hypothesis was tested that larvae compete with manure microorganisms for digestible carbohydrates added to fresh chicken excreta. This was done by adding gelatinized corn starch (digestible carbohydrate) or fructo-oligosaccharides (non-digestible carbohydrate) to unsterilized (active microbiota) and heat sterilized (inactive microbiota) excreta at 18% of substrate dry matter (DM), followed by inoculation with housefly eggs. Sterilized excreta with starch yielded the highest individual (13.2 mg) and total larval biomass (9.7 g) and highest nitrogen bioconversion (9.9%), with unsterilized pure excreta being second best (8.1 mg, 7.2 g and 9.3%, respectively). Starch supplemented unsterilized excreta and fructo-oligosaccharides supplemented excreta (sterilized and unsterilized) showed much lower larval biomass compared to pure unsterilized excreta. The experiment also showed that, though larval biomass increased, larval survival was negatively affected by adding starch to sterilized excreta. In **Chapter 3**, the dose-response relation between starch concentration and larval growth, survival and substrate bioconversion was determined for sterilized and unsterilized excreta. A starch content of 15-20% of DM yielded the highest overall mean total larval biomass (39 g) in unsterilized excreta, whereas 10% starch content led to a higher total larval biomass in unsterilized excreta (43 g). After their maxima at these concentrations in both types of excreta, total larval biomass decreased, much more gradually in sterilized excreta. Larval survival was highest in pure unsterilized excreta (80%) and decreased more or less linearly with increasing starch content to 14% at 50% starch, showing a similar decrease in sterilized excreta. The optimal starch contents increased nitrogen bioconversion with 40% (unsterilized, from 10.7 to 15%) and 100% (sterilized, from 7.5 to 15%) compared to pure unsterilized excreta. Around 20 to 25% starch content, uric acid and ammonia contents, pH, and C/N of substrate residues decreased rapidly, indicating a tipping point that occurred later in sterilized excreta. In **Chapter 4**, the theory developed in **Chapter 1**, that utilization of starch by larvae depends on larval stage (age) dependent amylases was tested. Starch and fresh excreta were added at 10% DM to unsterilized excreta, either at the start (0 h) or at 72 h during the larval growth period of 120 h, using

the extremely easily digestible glucose as a positive control (time effect starch specific) and indigestible cellulose as negative control (effect of stirring). Glucose and starch substantially improved larval performance compared with fresh manure and cellulose (non-digestible carbohydrate). Supplementation at 72 h, with glucose increased individual larval mass by 12%, total biomass by 33% and survival by 19% compared to 0 h. Starch at 72 h increased total biomass by 30% and survival by 24%, while effects on individual mass were small (2%). Dry matter bioconversion efficiency increased by 27% with late glucose and 20% with late starch, whereas late excreta addition caused an 18% decrease. Nitrogen bioconversion rose by 36% with late glucose and 37% with 72 h starch compared to 0 h. The results supported the hypothesis that starch is better utilized by older larvae than by younger larvae. The mechanism underlying the differences in larval biomass is that larval survival is higher when starch is added later, with the increase in total larval biomass driven by improved survival and not individual larva mass. In **Chapter 5**, a second theory described in **Chapter 1** was tested, addressing the hypothesized positive effect of higher larval densities on larval survival and early growth in starch-supplemented unsterilized excreta. Starch tends to lower larval survival and higher larval densities might mitigate this through more efficient digestion by increasing temperature and substrate mixing. Unsterilized excreta substrates, unsupplemented or containing glucose or starch at 10% of DM were inoculated with four egg densities (8-12-16-20 eggs/g substrate), with different replicates harvested at two harvest times (58 and 120 h). Higher densities improved survival of the early-stage larvae, particularly in supplemented substrates, but accelerated substrate drying reduced late-stage larval growth and bioconversion. Starch lowered residue ammonia and pH, suggesting better nitrogen retention. Statistical analysis showed that biomass yielded by early-stage growth was more associated with substrate, whereas late-stage biomass was more associated with density. Harvesting the larger biomass of early growth showed the nitrogen bioconversion, with gains of 13 to 22%. In **Chapter 6**, the results of the experimental chapters are evaluated, indicating challenges and potential improvement of experimental procedures, as well as implications of the thesis results for housefly mass-rearing and suggestions for future research. The **Appendices** describe developmental work on housefly rearing, egg collection and larvae harvesting. The main conclusions of this thesis are that housefly larval growth on and bioconversion of chicken excreta is limited by low availability of digestible energy. Moderate supplementation with easily digestible carbohydrates, particularly starch, substantially increases larval biomass and nitrogen bioconversion. However, starch supplementation reduces survival and its benefits are strongly constrained by competition with manure microorganisms, making microbial activity a key determinant of larval access to energy. Larval utilization of starch appears to be developmentally regulated, with older larvae benefiting most from supplementation. Higher larval densities modify growth and bioconversion outcomes by enhancing early survival but constrain late-stage growth by increased dehydration of substrates.

Acknowledgements

First, I would like to thank my supervisors, Dennis, Guido and Wouter. For hiring me, an unusual older PhD candidate, in the first place. For steering me into thinking in terms of nutrition, for your valuable insights, for your guidance in the academic and scientific process. For always responding quickly to my needs and requests. And for your flexibility and patience, conducting experiments went smoothly but finalizing the actual thesis took longer than needed, or as long as needed given the circumstances. Discussing experiments and the relation between them as common thread of the thesis with the three of you was always highly educational. One of the highlights of these meetings was Wouter letting me ramble about what I wanted to do for 30 minutes, only to say "I want a single hard hypothesis", making most of my proposed ideas obsolete. Only after I did all the experimental work I realized that, generalizing, a lot of experimental animal data are so small that you can only use them for testing exactly this, a single hard hypothesis. It might be the other way around though, with data difficult and expensive to obtain, a single hard hypothesis only requires small data.

Following ANU PhD courses was great fun and I learned quite a bit of really applied biochemistry and how to do experiments with livestock. Walter, Sonja, Wilbert, Thomas and others that I am forgetting were good teachers. Most courses outside ANU, done as part of the training program were also really interesting and sharpened my thinking in sometimes unsuspected ways. A special thanks goes out to Erika and Saskia, without whom all the chemical analyses would have been impossible. The ANU lab is just great, one of the better university labs I have seen and I have seen a few. In this context, I'd like to thank Niels for interesting discussions and answers, you really know your stuff. Special thanks also apply to Yvonne and Betty, who were essential for me, and most other people, for daily functioning at ANU. Talking with you was always a pleasure.

Talking about good facilities, I really enjoyed my time at Carus, washing, weighing and freezing my thousands of larvae surrounded by pelleting machines, ovens, tractors and other big hardware. I have to thank Tamme for showing me where everything was and solving practical problems. I also have to thank the staff at Radix Clima for hosting me and my flies at their plant facility. I am happy that most of you took the nuisance of escaped flies more or less lightly. We share the interest for growing plants and I always enjoyed talking to you and asking what you do. A special thanks to Pieter of ENTO, I enjoyed our smoking breaks and your pragmatic tips on insect cultivation methods.

My thanks also go out to all the people-related-to-flies that I met at consortium meetings or as fellow students. Esther, Giacomo, Francesco, Jacintha, Ida, Leo, Ljubinka, Ruilong, Roger, Walter and Xiaoqi. All of you made a lovely mix of socializing and fly-related discussion and with some of you, pretty intensive hours of fly experimentation.

Of course a major thank you goes to Angelès, who always supported me doing this and who was always there in the good and bad times in the years in the Netherlands.
I would also like to acknowledge the role of the houseflies used in this work, without which this research would not have been possible. Of all the animals I have experience with, the housefly was

among the most challenging in the amount of technique and management needed. I never really thought much about houseflies, or about any other diptera, but now that I have interacted so closely with them and their *in-vitro* peculiarities, I appreciate them.

About the author

Curriculum Vitae



Born in Delft, August 14 1973, Lennard completed his pre-university education (VWO) in Delft as well. In 1992 he started studying Biology at Leiden University, choosing the population biology/ecology specialization. He wrote two thesis, one on the ecology of stingless bees involving field work in Costa Rica for Utrecht University and one on naturalizing and invasive plant species in the Dutch flora. After graduating to Masters in 1998 he led a wandering working life, from tour guide for the then new Naturalis museum and biologist for the waterboard to working as technical assistant at the chemistry faculty of Leiden University and pharmaceutical companies. This was followed by a return to student life in 2004, for two years at the University of Amsterdam, focusing on epidemiology and writing another thesis on Lyme disease genotypes in ticks. After that he worked three years as a researcher for the Dutch Bee Research Centre, on statistical analysis of international honey bee loss data and epidemiology of honey bee pathogens. This line of work was continued when he moved to Utrecht University in 2013, with more focus on the environmental impact of pesticides. When this ended, he spend a year at the national Institute for Public Health and the Environment (RIVM) as a junior epidemiologist at the Gastro-intestinal and Zoonotic disease department before moving to the South of France in 2016. After working as a statistician for a support platform of Montpellier University, circumstances drove him back to the Netherlands where he applied for the PhD position at Wageningen University. In his free time, he enjoys exercising his greyhounds, beekeeping, aquarium keeping, gardening and rearing tropical mantises and spiders.

Training and supervision plan[1]

Education and training	Year	ECTS[2]
The Basic Package		
WIAS Introduction Day (mandatory)	2018	0.3
Course Ethics and Animal Sciences	2020	0.8
Course Scientific Integrity	2020	0.6
Course on essential skills	2019	1.2
Disciplinary Competences		
ANU-30806 Animal Nutrition and Physiology	2020	6.0
Develop you change agency and valorise your potential impact	2022	2.0
Literature survey "Mass culture of houseflies on livestock manure"	2019	6.0
Professional Competences		
Course The Final Touch	2023	0.6
Entrepreneurship Inside and Outside Science	2022	0.8
WGS PhD Workshop Carousel 2023	2023	0.3
Project and Time management	2020	1.5
Societal Relevance		
Societal Impact of your research	2022	1.5
Presentation Skills		
WIAS Science Day, Ede, the Netherlands (oral)	2021	1.0
INSECTA 2021, Magdeburg, Germany (oral)	2021	1.0
IOBC-MRQA Workshop, Bologna, Italy (oral)	2022	1.0
INSECTA 2022, Giessen, Germany (oral)	2022	1.0
Teaching competences		
Supervision Introduction to Animal Sciences	2019	0.5
Supervision Introduction to Animal Sciences	2021	1.1
Assisting rumen practical Biology of Domestic Animals	2022	0.1
Supervising 2 MSc students	2020-2023	3.0
Education and training total ECTS:		30.3

[1] Completed in fullfilment of the requierments for the educational certificate of the Wageningen Institute of Animal Science (WIAS). [2] One ECTS credit equals a study load of approximately 28 hours.

Appendix 1 buoyancy separation of larvae

Separation of housefly larvae from their substrate is a necessity for bioconversion studies. Manual separation, searching through the substrate and collecting individual larvae by hand, is time consuming and impossible to be conducted on a large scale. Additionally, when the larval substrate is animal manure, safety is a concern (pathogens) and contact with larvae and substrate is best kept to a minimum.

Living larvae are difficult to manipulate, they are small, often numerous and when wet/moist they adhere to smooth surfaces (plastic, inox) and can move relatively fast. Also, some substrate particles tend to cling to larvae, inducing the need for a post separation cleaning step (i.e. let larvae move on/between clean tissues).

This text describes the development of a suitable separation method.

Unsuccessful attempts

To avoid manual separation, different methods were tested with very limited success. These were:

1. Heat from below. In theory higher temperatures drive larvae out of the substrate. When heat (50°C) was applied below the substrate by a hot plate with a thermostat, it was visually observed that about half of the larvae moved to the surface in the course of one hour. Possibly this method would work when heating time is applied for >3 h. However, a relatively large number of dead larvae were found after the 1 h test. A problem with this method is that larvae are photophobic, moving upwards to escape heat is against their natural behavior, which leads to low recoveries. In addition, the larvae still needed to be collected with tweezers from the substrate surface with most larvae still having substrate particles adhering to them. The latter process is difficult to scale up.
2. Cold from below. Similar to heat from below, in theory low temperatures drive larvae out of the substrate. It might be less destructive for the substrate than heat from below. In the test performed, having containers floating in ice water, recovery after 2 h was less than that with heat from below and the other drawbacks also apply: larvae do not want to move to the surface, the need to pick them up and the need to clean them. Scaling up would be easy, multiple containers floating in ice water but not worth it due to the duration and low recovery.
3. Carbon dioxide (CO_2). In theory CO_2 would cause larvae to move out of the substrate as oxygen levels will decrease. When a container was flushed continuously for 30 s with pure CO_2 and applying a non-ventilating lid, only a few larvae close to the surface appear with the majority of the larvae dying in the substrate over the next 15 min. Cickova *et al*. (2012) mention a variant of this technique, where they just cover the substrates with lids, but they also found that recovery is low and mortality is high.
4. Electricity. In the hope that larvae would react similarly to earthworms, who rapidly come to the surface when an electrical field is applied, a 10V 25mA charge was applied to inox plates pushed into the substrate (Photo 1). Larvae show a stress response. They start to move

erratically and turning around their longitudinal axis, but they do not move up to the substrate surface.

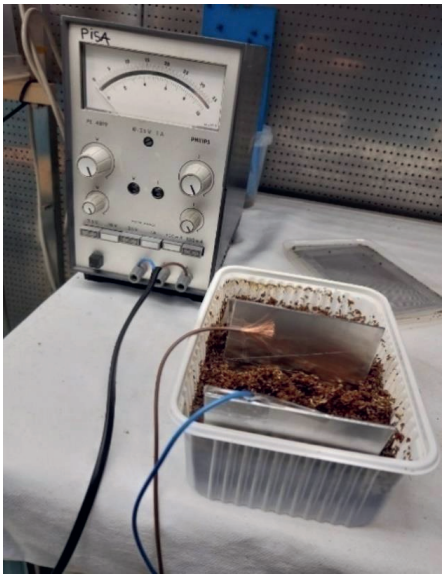


Photo 1. Applying DC current to bran substrate.

5. Wet sieving. In theory the larvae can be washed from their substrate and collected in a sieve with the right mesh size. In practice the use of sieves leads to a wet mass of larvae that contains manure particles as big or bigger than the larvae. Also, the larvae-manure mixture sticks to the sieve and while the larvae are quickly spreading and getting out of the sieve, the mixture needs to be removed by forcefully hitting the sieve upside down on a hard surface after rinsing the larvae-manure mix into a corner of the sieve. Manipulation of the moving live larvae mass on a paper towel is difficult. Although feasible, the separation of larvae and manure particles in practice remains a challenge.

6. Light and heat. A promising method is the use of light and heat from above. This method is commonly used to sample arthropods from soil (Tullgren funnel). The substrate is spread on a sieve and light and heat are applied by means of a lamp at some distance. In older literature, several designs specifically made for the purpose of separating *Musca* and *Fannia* larvae are described (Brydon and Fuller 1966). This method fits to the natural tendency of larvae to move down to avoid light and heat, but the method is time consuming (separation takes hours according to the literature, systems run overnight) and multiple sample set-ups are rather voluminous. A small prototype with 10 Watt led spots I constructed did not work well. Only small amounts of substrate could be used and larvae took indeed hours to leave the substrate for good recovery.

Buoyancy separation of larvae.

After the unsuccessful attempts described above, I conceived the idea that separation by differential buoyancy might be a feasible option. In pure water, chicken manure and larvae sink to the bottom of the container. I had the idea that in water containing a higher concentration of NaCl, larvae might float due to the difference in density. The larval cuticle is wax-like, has low wettability, causing water to bead and larvae are not penetrated by the salt solution right away. Manure on the other hand becomes wet and sinks. I conduced an initial test in 2019 and results were highly promising.

I made a 5, 10 and 14 % solution of NaCl (basic kitchen salt) in water using standard 1 L pots: 25, 50 and 70 g NaCl/500 ml. Solutions were slightly opaque, probably caused by an anti-caking agent.

To each container a tablespoon of chicken manure was added which contained 50-100, 4 d old larvae. As expected, in pure water both manure and the majority of larvae sank to the bottom (Photo 2). In the 5% solution, considerably more larvae floated (Photo 3), while in the 10 and 14% solutions nearly all larvae floated on top of the solutions (Photo 4 and 5, respectively). The particles floating around with the larvae were predominantly bran, which was present as it was used to inoculate the manure. Chicken manure sank in all solutions.

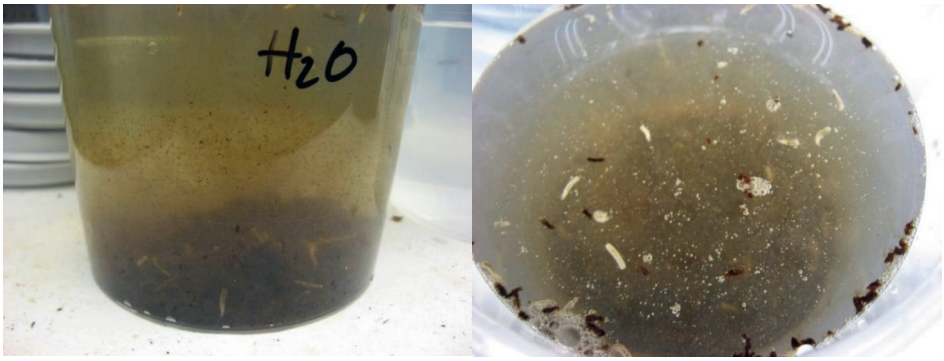


Photo 2.Chicken manure with larvae in pure water. Left: side view, right: surface view.

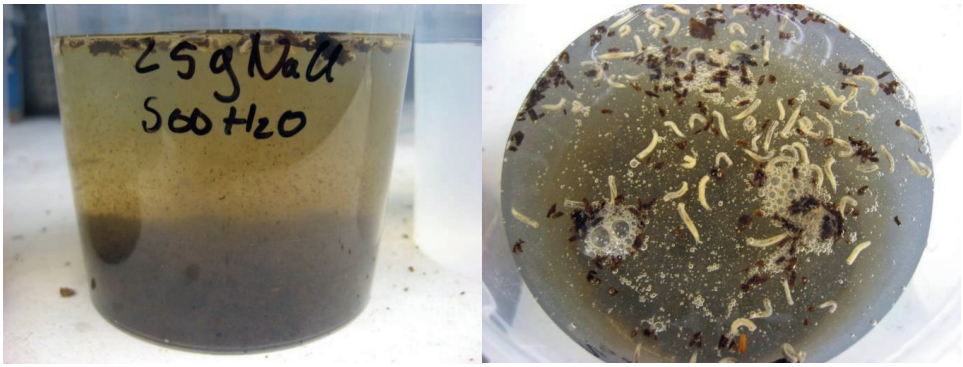


Photo 3.Chicken manure with larvae dissolved in 5 NaCl %. Left: side view, right: surface view.

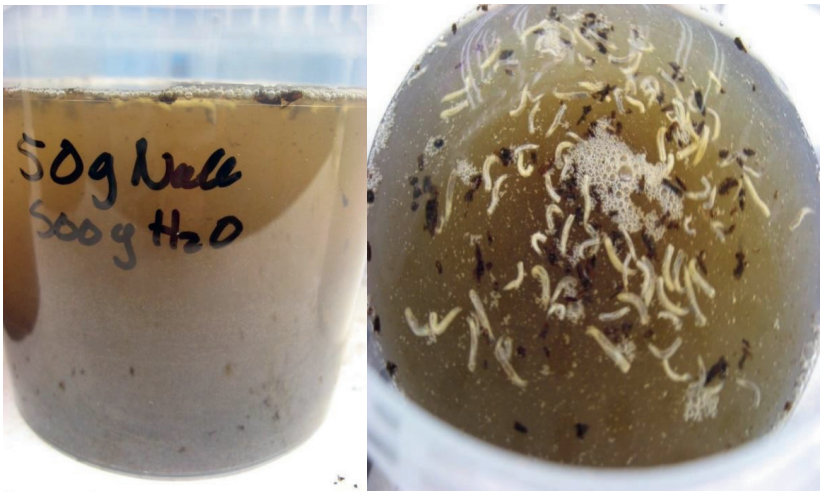


Photo 4.Chicken manure with larvae dissolved in 10 NaCl %. Left: side view, right: surface view.



Photo 5.Chicken manure with larvae dissolved in 14 NaCl %. A. Left: side view, right: surface view.

In this quick experiment, a dose-response (salt % vs larvae floating) could not be derived as the crudely taken samples contained different amounts of larvae and the evaluation was based on visual inspection only. But the principle appeared to work, with 14% salt addition, the recovery was very good. All solutions were decanted into a similar container without dripping or splashing and the surface layer with larvae can be perfectly separated from the manure residue. Decanting can furthermore be achieved in a matter of seconds.

Post decanting, most larvae in pure water remained with the manure. For the 5% NaCl solution, some larvae remained with the manure and for the 10 and 14% solutions only 1-3 larvae remained in the residue. These last 1 to 3 could be easily collected from the manure by hand.

All solutions were left for 1 h to observe effects of salt on the larvae. Interestingly, larvae in the pure water residue all died, presumably by drowning (Photo 6). Larvae floating at the surface of decantated solution were all alive after 1 h (Photo 7).

The larval mass of 14% NaCl was poured through a primitive mesh sieve to see how to further clean the sample. Bran and other particles indeed remained on the mesh but collecting the wet and mobile larvae proved to be difficult. Collected larvae were put on bran substrate to check for influence of salt treatment on survival. No difference in development of pupae to were observed compared to "unsalted" larvae.





Photo 6 (left). Dead larvae and manure residue in pure water. Photo 7 (right). Live larvae in 14% NaCl.

Further notes (2023) after applying the method in experiments.

The method describe above was highly successful in my experiments (**Chapter 2**, **3**, **4** and **5**) and those of other students separating larvae from chicken excreta. A 15% salt solution also worked well and the method was easily scaled up to process manure masses of 5 kg. For larger volumes, it is recommended to use square plastic boxes rather than pots/cylinders as they are easier for collecting larvae from the surface. An extra washing step, so rinsing larvae in salt water once more provided an even cleaner larval mass. A three step version where larvae are briefly washed in pure water after two salt water washes provided an even cleaner product as larvae sink and some debris will float and can quickly be decanted off. After separation, the best procedure was to transfer the live larvae in a pot containing tissue paper to absorb moisture. Larvae can then be euthanized and stored by freezing. After thawing, larvae can be further cleaned from debris by placing them on a large sheet of rough (tissue) paper and gently agitating the paper sheet. Larvae clump together while debris does not stick to larvae much and "rolls" off the sheet. Photo 8 and 9 show typical larger scale use of the method, while Photo 10 shows larvae after two salt water and one tap water washings. The large amounts of salt water-manure residue are a problem to dispatch. A good method is collecting all this material in a large container and let the manure sink to the bottom. Water can then be removed from the mix.

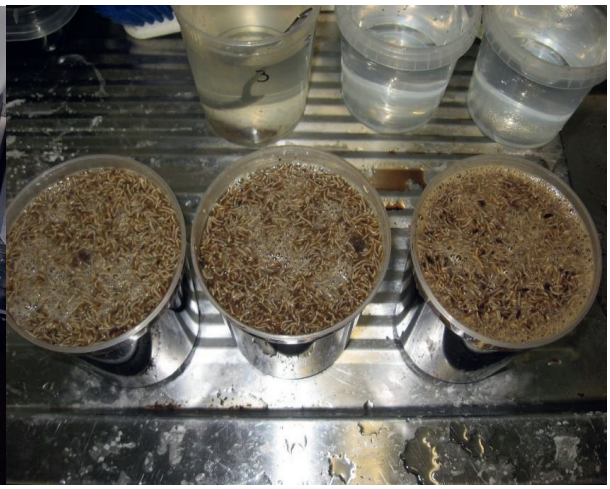



Photo 8 (left). Larger scale. Photo 9 (right). Washing in series.



Photo 10. Cleaner larvae after two salt water and one tap water wash.

References.

Brydon, H. W., Fuller, R. G. (1966). A portable apparatus for separating fly larvae from poultry droppings. *Journal of Economic Entomology*, 59(2), 448-452.

Čičková, H., Kozánek, M., Morávek, I., Takáč, P. (2012). A behavioral method for separation of house fly (Diptera: Muscidae) larvae from processed pig manure. *Journal of Economic Entomology*, 105(1), 62-66.

Appendix 2 housefly culture and egg harvest

Rearing houseflies to obtain large numbers of eggs at a predetermined time is a challenge and maintaining a large housefly culture requires dedicated care. Larvae are often reared on mixtures of bran, dried yeast and milk powder. An alternative "dog kibble" substrate is described here. The dog kibble substrate involves less ingredients and mixing while yielding more larvae per gram substrate. It also largely solved the problem of substrate drying out in strongly ventilated climate cells, as the separate bran layer prevents drying of the wet dog kibbles underneath.

1. Caging adult flies

A climate room for houseflies requires temperatures between 23 and 35 °C. Temperatures at the lower end of this range lead to longer development times and longer lifespans. Humidity can be 50 to 80% while daylight hours can range from 8 to 16 h.

1. Place a well-cleaned Bugdorm cage or equivalent in its final position. Furnish the cage with cardboard on the cage floor (for easier cleaning) and hang several large cardboard strips in the cage for the flies to sit on (Photo 1).This surface enlargement allows for more flies to be maintained. Cardboard egg crates are also a very good way to enlarge the surface even more.
2. Place containers with pupae ready to hatch in the cage.
3. Place sugar, feed and water containers in the cage.

Notes: flies defecate and spit on cage surfaces a lot. Clean dirty Bugdorm cages by wetting with lukewarm water (hot water damages the transparent plastic sides) and softly sponge them with dishwashing liquid soap or another neutral detergent. Rinse well and air dry. The Bugdorm cages used can house up to ~5000 flies maximum with optimal furnishings. Ideally, the cardboard furnishing should create spaces from where the flies see the least movement of the operator. Very full cages of flies can "panic" from visual disturbance, with all the flies flying into a corner, leading to damaged flies. Always try to move slow in front of high density flies and place objects in their cages slowly.

2. Feeding adult flies

1. Providing sugar: place ~100 g of regular sugar cubes (sucrose) in a plastic tray. Positioning a colored marker in the tray is beneficial, flies learn, but this is not needed (Photo 2).
2. Providing feed: baby formula (alternative: skimmed milk powder). Cover disposable paper or plastic plates with a thin layer of powder. Two to three table spoons per tray, well spread out (Photo 3). Dense cages require multiple plates to allow stress free access to all flies.
3. Providing water: make a small slit in the lid of a polypropylene pot and insert a piece of cotton fabric (a wick), part of it protruding from the lid surface, part of it to be submerged in the water. Fill the pot with water and close the lid. Lay 1-3 cotton pads on the fabric to create a drinking surface (Photo 4a and b). Dense cages require multiple pots to allow stress free access.

Notes: feed as much as flies use in 24 h and replace the plates when moist and/or dirty (brown yellow film). Flies require to be fed fresh powder at least every 48 h, daily feeding of smaller quantities is advisable. Greater numbers of flies require multiple plates to allow individual flies to eat stress free. Flies can also be provided deactivated yeast. This is highly nutritious and flies like it a lot. Flies also like apple and sweet fruit pulp. Monitor the water pots closely and replace when 50% of the water is lost or when the surface turns dirty (slimy/yellow). Replacing water every 48 h is a good practice, gravid flies without oviposition substrates will readily oviposit on dirty water pots. This needs to be avoided as it hinders egg collection. The more flies there are in a cage, the more important feed supply and feed hygiene is. Dirty or hungry flies die quickly.



Photo 1 (left). Cage cardboard furnishing. Photo 2 (right). Sugar cube feeder.

3. Euthanizing adult flies

After 1-3 egg harvesting sessions over the course of a few days, the egg laying capacity of flies decreases. Offering direct oviposition substrates, described below, at this time makes enables the females to lay their last eggs that can be used to continue the population. After this, flies can be euthanized to prepare the cage for another generation. Euthanasia is most humane by putting the cage in a freezer. Second best method is to remove sugar and water, this kills nearly all flies in 2 days.

Notes: males die easier/faster than females. The last living flies in a cage are always females. With practice, sexes can be distinguished. Females tend to be larger, have wider abdomens and have more space between the eyes compared to males.



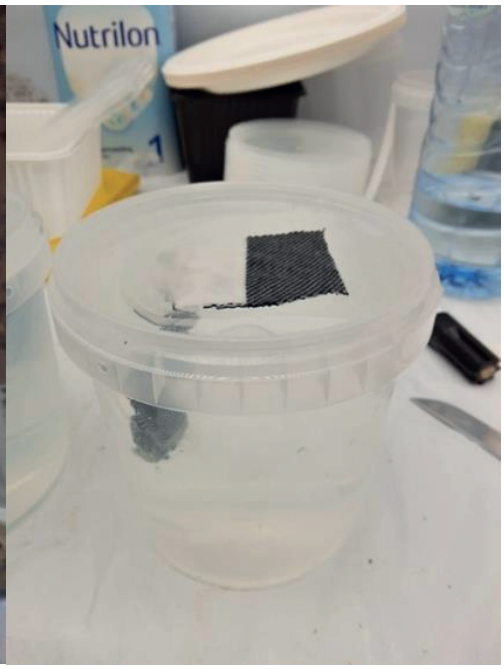


Photo 3 (right). Flies on baby formula. Photo 4a (left). Cotton cloth wick in watering pot.





Photo 4b (left). Finished watering pot with cotton pads. Photo 5 (right). Modified sticky traps.

4. Trapping adult flies

Working with large numbers of houseflies without any escaping is very difficult if not impossible. Houseflies are extremely agile and freely manoeuvre in closed spaces (in contrast to black soldier flies). Even with practice brought on by experience, a room with several cages at peak density (~20000 flies) will have dozens of escapees. Errors like forgetting to properly close cage sleaves or

holes (seams coming apart) will lead to hundreds of escapees. Escapees easily make their way outside the climate room into the rest of the building (toilets) which leads to people becoming less tolerant to house flies (and their operators). Setting traps and additional use of electric fly swatters must be a serious and continuous effort. Traditional sticky paper traps should be hung everywhere possible, above trays with larvae, in corners and along the ceiling. The plastic blue/yellow sheet sticky traps work best when modified with small black pieces of plastic (this was found to attract more flies) and attached to a bait (pot with larval substrate, Photo 5). Flies tend to aggregate at the coolest spots in the climate room and traps are best placed there. Having 6-10 active fresh traps in a room at all times is good practice.

5. Rearing larvae to obtain adults

After 7 to 10 days at 25-28 °C, adult flies will lay eggs. The easiest way to continue the culture is to have flies oviposit directly in suitable larval substrate and partition young larvae onto fresh substrates. Methods that collect eggs to maintain the culture introduce extra work and increase larval mortality.

Notes: Flies kept at lower temperatures and lower densities live and lay eggs longer. Gravid females of the right age have yellowish white abdomens, abdomens of young females are greyish white and smaller. Oviposition trays with large numbers of larvae generate a lot of heat. This is normal and probably beneficial for larval growth.

Oviposition substrates

1. Add (~100 g) Albert Heijn brand dog kibble or equivalent to a plastic tray (800 ml “nasi tray”).
2. Add (~250 ml) water and let it soak for a few minutes.
3. Stir in (~100 g) wheat bran. Take care to have some kibbles exposed to the air. Add 50 to 150 ml extra water to moisten the surface (Photo 6).
4. Cut up a few small black plastic plant pots and put the pieces on the bran, creating “dark crevices” and place tissue paper or coffee filter extending outside the tray to make a ramp for flies to easy walk in and out (Photo 7). Easy access is important, flies need to get out easy. Flies that cannot get out get dirty and die.
5. Cover the tray with aluminium foil, to prevent evaporation, leaving a wide slit (2 - 4 cm) at the side with the ramp. Extra black cloth or plastic attracts flies (Photo 8).
6. Place the tray in the cage and leave it for 24 -72 h.
7. Put a lid of sorts on the tray and take it outside (out of the building) to remove the flies inside the tray.
8. With a spoon, scoop substrate with larvae into fresh substrate trays. Take care to include larval aggregations at the bottom of the tray (Photo 9).



Photo 6 (left). Mushy exposed dog kibble with wheat bran. Photo 7 (right). Interior of tray with artificial crevices and a tissue ramp.



Photo 8 (left). Finished oviposition tray exterior. Photo 9 (right). Typical mass of young larvae in the oviposition tray.

Follow-up larval substrates

1. Put 1 cup (~100 g) Albert Heijn brand dog kibble or equivalent in a 800 ml “nasi tray” or preferably a higher 1800 ml “black tray”.

2. Add 1 cm of water (~200 ml) and stir to loosen and flatten the kibble mass. Let it rest for a few min, partly soaking up the water.
3. Add 1.5 – 2 cups (250 – 300 ml) wheat bran on top of the kibbles. Spread to a uniform layer. Do not mix, the dry bran serves first as a cover for the wet layer to prevent drying out.
4. In a corner of the tray, with a spoon move the bran a bit into the wet kibble creating a small wet corner.
5. Add young larvae from oviposition substrate in this corner (Photo 10). Larvae will move down into the wet dog kibble, finding their ideal moisture. They will mix in and use the bran when growing.
6. Try to aim for a high density, ~6 larvae/g. This is difficult to gauge but it comes down to dividing an oviposition tray with 2 day old larvae into 4 or 5 new trays with fresh substrate.
7. Pick up the trays and feel their weight (this will lead to insight on adequate moisture under specific ventilation circumstances). Push some bran aside with a spoon to check larvae. When light they are too dry (powdery texture at the bottom of the tray) add extra water, ~100 - 200 ml. Less is more, too wet and larvae will escape.
8. Keep on checking dryness of larvae substrates for the next 4 days, add water when needed.
9. When larvae appear wriggling in clusters at the surface of substrates that do not feel dry, they are hungry. Substrates can be supplemented with soaked dog kibble. A few tablespoons at the surface is fine, larvae will dig it in.
10. When big larvae are pupating, no extra water is needed, substrate needs to dry out.
11. Well-fed larvae pupate at day 5-6. Sooner in dry/dried out substrates, yielding smaller pupae.
12. Place trays with pupae in a suitable cage before pupation.
13. Note: when (surface) substrate humidity is optimal, there is no need to cover trays with a (gauze) lid. Dry walls will prevent dry larvae climbing out. Wet larvae do climb the walls, this is a sign the substrate is too wet. Wetness can be remediated by adding extra bran on top.

Notes: often methyl-paraben (nipagin) is added to bran substrates to prevent mold. There is no need for it with the above described method. Not using eggs but transfer of older larvae at high density, bacterial symbionts and possibly heat production prevent molds. Probably by producing ammonia, good substrates with lots of larvae always smell to ammonia, bad substrates with few larvae smell putrid and can develop a white mold when moisture is high enough.



Photo 10 (left). Inoculated kibble trays. Note the wet substrate in tray corners, the presence of traps and the absence of lids. Photo 11 (right). Large presence of gravid females.

6. Harvesting housefly eggs

Timely planning should lead to cages with well-fed adults of the proper age, 7 but better 10-13 days after eclosion at 28 °C. Presence of many females with big yellowish white abdomens (Photo 11) is a sign that this is the case. In the days before the planned egg harvest, monitor the watering pots for egg laying females. Females that need to lay will do so when the water pot cotton pads become the slightest bit dirty. Replacing water pots daily or more prevents this but not fully. The egg harvesting "sock" procedure (see below) leads to clean, dry eggs that are easily harvested. Flies will need 3 to 6 h to lay eggs in the socks. Optimal cages with 3000-4000 adults can lay 3-5 g of eggs per sock. Offering multiple socks (3 or more) prevents crowding and stress. Always plan to have surplus eggs, if not needed they can be discarded or used for extra larvae. Socks can be offered to flies for 2-3 consecutive days but the amount of eggs will decrease after the first day.

Making oviposition socks

1. Leave pasteurized milk (any supermarket brand will do, I used semi-skimmed) in a clean glass pot at room temperature ~24 h before making the oviposition medium.
2. Fill a ~300 ml wider than tall plastic pot halfway with the day old milk. Add a drop of household ammonia and/or ~20-100 ml of a watery extract of old larvae medium to the milk.
3. Cut a clean (no detergent or perfume odor) black cotton sock and fit it to the pot, spanning the surface and the tip hanging a few cm in the milk. The sock will wicker the milk and provide a moist environment for the eggs (Photo 12).

4. Cut a few small pieces of the sock and wet them with milk. Put the wet pieces into the cavity formed by the sock, as to make a few “strategic” crevices (Photo 13).
5. Make a lid of aluminium foil that covers the rims of the sock, as to prevent evaporation and make the cavity dark. Take care the lid has a slit 5-10 mm wide at a side to allow access (Photo 14).
6. Place the pots in a cage with flies of the proper age for 4 to 8 h. The shorter, the more similar the age of the eggs but the larger the amount of females needed.
7. Monitor the pots every few hours for the milk level, if it drops more than 10%, add extra milk or water.
8. Remove the pots from the cage, place it in a big container with lid to prevent females escaping. Remove the females in the climate room with tweezers or do this outside (the building). The result of an optimal sock run will look like Photo 15.





Photo 12 (left). Milk sock side view. Note the trap. Photo 13 (right). Sock interior, extra pieces of soaked cloth forming crevices.



Photo 14 (left). Milk sock with foil cover ready to use. Note the slit for access of flies. Photo 15 (right). Successful sock with dry eggs.

Weighing eggs

Eggs are partitioned onto substrates by weight, with the number of eggs based on individual egg weight estimation. Conducting a weight-count check before an experiment is essential to obtain accurate results. Weigh a few clumps of eggs and count the eggs to derive the weight per egg. This should be 0.06-0.08 mg per egg, possibly varying with moisture. Small portions of eggs, <100 mg, are more difficult to weigh because eggs tend to clump together. If possible, try experiments on a scale that allows the use of 200-300 mg total egg weight, adding or removing clumps to/from this weight is much easier and decreases weighing and manipulation errors. Proper tools for manipulating eggs are tooth picks (wetted), plastic micro lab spoons and strips cut from plastic polypropylene (nasi tray lids). Eggs are best weighed on small pieces of filter paper or aluminium foil. Prior to use, papers with eggs are best stored in a closed container with water sprayed on walls and wet tissue on the bottom to generate high air humidity. Practicing weighing eggs before an official experiment is essential.

Weighing procedure

1. After removal of (most) flies, open the sock to get to the eggs (Photo 16).
2. The best strategy is to gently break up large clumps of eggs into several clumps, eye-gauged to be a bit more than the weight needed, on the micro spoon/piece of plastic and small clumps onto the filter paper until the desired weight is reached (Photo 17).
3. Do not worry if the exact amount is not achieved at first, especially with amounts <100 mg. Within 2-5% of the target weight within 5-10 min is doable for >100 mg egg amounts with experience.

4. Apply the eggs to the experimental substrate in a “crevice” present or made by pushing with a spoon. Tumble the eggs in the crevice and place the filter paper on the crevice. A little misting with water to wet the paper is probably beneficial.

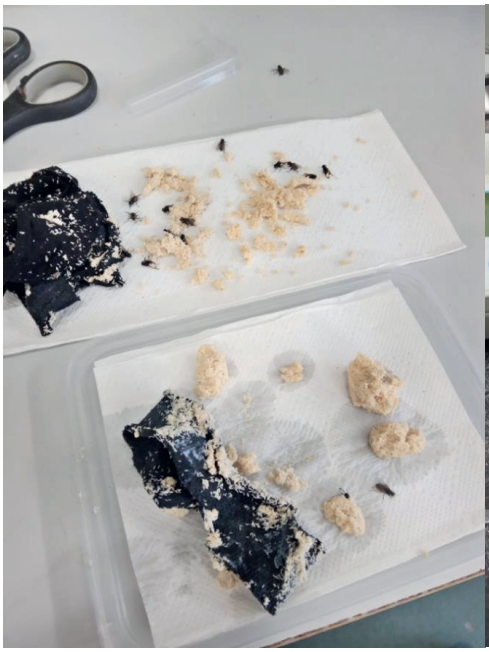




Photo 16 (left). Eggs ready for weighing. Photo 17 (right). Portions of eggs.

7. Mites in housefly culture and chicken excreta

Two types (species) of mites were relevant during the housefly bioconversion experiments. The first are grain mites (Photo 19), possibly genus *Acarus*, that most likely come with larvae in bran, bran itself or with mealworms in bran. Grain mites can build up to large populations in and under cages and spread throughout the climate cell. If very high densities are present they cling to adult flies, hindering them but it is unclear if they do any damage. If mites are seen, bran can be frozen to kill these mites and the climate cell, fly cages and other hardware should be thoroughly and repeatedly cleaned with water with detergent. If mites are visible in the substrate of acquired larvae, larvae can be separated from their substrate by hand and rinsed repeatedly with room temperature water (larvae sink, mites float), after which they can be put on mite free substrates. The second mite is a bird mite (Photo 20), possibly genus *Ornithonyssus,* that is substantially larger than a grain mite. Fresh chicken excreta can have high numbers of these mites, that will move out of uncovered containers into cars and onto people manipulating the excreta. These mites can bite humans and are highly unpleasant. Excreta should be transported in well closed containers and it is recommended to have a spray bottle with a water-detergent-alcohol mix to spray and wipe containers before loading them into a car. Freezing excreta kills mites quickly. When manipulating large amounts of unfrozen fresh excreta, a coverall, spare clothes and showering right afterwards are good practice.



Photo 19 (left). Grain mites. Photo 20 (right). A chicken mite.

Colophon

Cover photograph: Yavanna Aartsma (used with permission)

Cover design: L.W. Pisa

Printed by: Proefschriftma